PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/62, 15/31, C07K 14/245, 14/575, A61K 38/22, 39/108

A1

(11) International Publication Number: WO 98/21344

(43) International Publication Date: 22 May 1998 (22.05.98)

(21) International Application Number: PCT/US97/20584

(22) International Filing Date: 12 November 1997 (12.11.97)

(30) Priority Data:
08/747,410 12 November 1996 (12.11.96) US

(71) Applicant: MICHIGAN STATE UNIVERSITY [US/US]; 238 Administration Building, East Lansing, MI 48824–1046 (US).

(72) Inventors: BAGDASARIAN, Michael; 1141 Woodwind Trail, Haslett, MI 48824 (US). IRELAND, James; 2805 Brentwood Avenue, East Lansing, MI 48823 (US).

(74) Agent: BRUESS, Steven, C.; Merchant, Gould, Smith, Edell, Welter & Schmidt, P.A., 3100 Norwest Center, 90 South Seventh Street, Minneapolis, MN 55402–4131 (US).

(81) Designated States: CA, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: CHIMERIC LTB VACCINES

(57) Abstract

A chimeric protein formed of a small peptide antigen inserted into an exposed surface region of the B subunit of heat–labile enterotoxin of *E. coli* useful as an immunogenic vaccine composition.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

CHIMERIC LTB VACCINES

Field of the Invention:

This invention relates to useful immunogenic molecules formed of the beta subunit of heat-labile enterotoxin (LTB) and an antigenic peptide antigen. More particularly, an antigenic peptide is genetically inserted into an exposed loop region of LTB, resulting in the production of a three dimensional molecule having the inserted antigen exposed on its surface.

Background of the Invention:

The development of vaccines based on small antigenic epitopes is hampered by the inability of the small antigen to elicit a good immune response in a host animal. The use of carrier immunogens provides some assistance in the immune response, but often decreases the specific activity and yield of the response against the desired antigen. Methods for conjugation of antigens to carrier agents are costly, and generally utilize hazardous chemicals. Covalent coupling of antigen to a carrier protein is inherently variable, resulting in an antigen with an imprecise structure, compromising vaccine potency. The use of adjuvants also tends to decrease the yield of specific antibodies and can be harmful to the animal host, causing abcesses, skin lesions, and hypersensitivity. These factors are unacceptable for the production of a commercially useful vaccine.

Chimeric molecules formed of large carrier proteins with attached peptide epitopes have been suggested as useful vaccines for small peptide antigens. However, added peptides extending from a three-dimensional protein are generally susceptible to proteolytic degradation. Insertion of an antigenic peptide into an interior portion of a carrier protein may avoid degradation problems, but disruption of the carrier protein's native sequence can alter the carrier's three dimensional structure and thus its function, including its ability to act as an efficient immunogen.

The non-toxic beta subunit of cholera toxin (CTB) and the related B subunit of heat-labile enterotoxin from *E.*coli (LTB) are powerful immunogens that have been suggested for use as carriers of foreign epitopes. In studies testing the activity of CTB, antigenic peptides have been genetically fused to either the N- or C-terminus and tested for activity. These constructs were generally susceptible to

rapid proteolytic degradation of the terminally fused peptide. (European Patent Application No: 89312713.4, published June 13, 1990.) In other studies, a CTB-peptide molecule having a 10 amino acid peptide from the HIV-1 gp120 envelope protein substituted for eight amino acids in CTB at positions 56-63 was shown to be resistant from proteolytic degradation as compared with an N-terminal CTB-peptide product. However, only a detectable response to the substituted gp120 epitope was obtained, and only in some, not all animal hosts. (Bäckström, et al., 1994, *Gene* 149:211-217.)

It is therefore highly desirable to develop an efficient and commercially useful process for producing immunogenic molecules containing antigenic peptide epitopes for use as vaccines, where the immunogenic molecule permits good recognition of the epitope as antigenic without high susceptibility to proteolytic degradation and produces a good immune response against the inserted antigen when administered to a host animal in the absence of adjuvant.

Summary of the Invention:

Chimeric molecules comprising the B subunit of heat-labile enterotoxin (LTB) and an inserted antigenic peptide have been found to display the antigenic epitope in an exposed surface of the LTB molecule without disruption of LTB folding and pentameric assembly and to provide immunogenic molecules useful in generating an immune response against the inserted small antigen. The LTB protein is also referred to as etxB (for enterotoxin B). This protein is encoded by the *etxB* gene.

Specific regions of the nucleic acid sequence encoding LTB have been identified as suitable antigen-insertion positions. A nucleic acid construct is produced having a nucleic acid sequence encoding the antigen inserted into the nucleic acid sequence encoding LTB. The insertion is made such that the expressed LTB-antigen fusion protein will include the inserted antigen in an external, exposed loop position. For example, when the antigen's sequence is inserted at approximately nucleotide 237 of *etxB* without loss of any LTB sequences, the expressed fusion protein displays the antigen on an exposed surface of the folded LTB molecule. At nucleotide 237, the *etxB* sequence contains a unique *Sma I*

restriction site. In a preferred embodiment of the invention, the antigenic peptide is inserted at the unique *Sma I* site.

In a most preferred embodiment of the invention, the antigenic fragment is a sequence of the αC subunit of inhibin, the fertility-modulating protein. For example, preferred antigens include bINHαC$^{1-14}$ and bINHαC$^{1-26}$ containing the first fourteen and first 26 N-terminal amino acids of the bovine inhibin alpha-C subunit, respectively. The antigenic sequence is inserted into the LTB molecule by inserting the gene encoding the inhibin fragment into *etxB* at the unique *Sma I* restriction site.

Since LTB is a pentameric molecule containing multiple exposed surfaces, the LTB:antigen fusion proteins, when used as vaccines, present multiple antigens for antibody development.

Brief Description of the Figures:

Figure 1 is a diagram showing the pMMB522 plasmid containing the *etxB:*bINHαC$^{1-14}$ gene sequences under the control of an IPTG-inducible *tac* promotor (p*tac*) from pMMB68, replication determinants of the broad host-range plasmid (rep), the gene encoding β-lactamase (bla), and the gene encoding the lac repressor (lacI^{Q}).

Figure 2 is a photograph of an SDS-PAGE gel comparing the electrophoretic mobility of wild-type LTB and the LTB-Inhibin fusion protein.

Figure 3 is a graph showing reactivity of the pentameric form of the fusion protein LTB:bINHα$_c^{1-14}$ in an anti-inhibin radioimmunoassay. The x-axis represents the amounts of protein in each tube. The units of protein amounts are nanograms for the gly.tyr peptide, and micrograms for the other peptides.

Figure 4 is a graph showing lack of reactivity of the monomeric form of the fusion protein LTB:bINHα$_c^{1-14}$ in an anti-inhibin radioimmunoassay. The x-axis represents the amounts of protein in each tube. The units of protein amounts are nanograms for the gly.tyr peptide, and micrograms for the other peptides.

Figure 5 is a graph showing anti-inhibin antibody titers in rabbits immunized with LTB-inhibin fusion protein.

Figures 6A, 6B and 6C are graphs showing anti-inhibin antibody titers in mice immunized with anti-LTB-inhibin fusion protein antibodies.

Figure 7 is a graph showing antibody titers in mice actively immunized with monomeric LTB-inhibin fusion protein.

Figure 8 is a graph showing antibody titers in mice actively immunized with pentameric LTB-inhibin fusion protein.

Figure 9 shows the published sequence encoding LTB.

Detailed Description of the Preferred Embodiments:

In the preferred embodiments of the invention, an immunogenic carrier molecule, the non-toxic B subunit of the *E.coli* heat-labile enterotoxin (LTB), is modified to include an inserted antigenic peptide. The inserted antigenic peptide is positioned in an exposed site of the LTB molecule, e.g., in an external surface of one of the molecule's loops, resulting in the presentation of the antigenic epitope on an exposed surface of the three dimensional chimeric molecule. When used as a vaccine, the chimeric antigen-LTB molecule is effective in eliciting an antibody response against the antigenic peptide in host animals. Vaccination of a host animal with the chimeric antigen-LTB results in the development of specific anti-antigen antibodies in the animal, preferably in the absence of added adjuvant.

LTB

Heat-labile enterotoxin from *E.coli* is a bacterial protein toxin having an AB_5 multimer structure. The B pentamer serves a membrane-binding function and the A subunit is needed for enzymatic activity. Structurally, the B subunits are arranged in a donut shape as a highly stable pentamer. The donut shape is formed from five of the identical B subunit monomers arranged symmetrically around a central 5-fold axis with a pore in the middle. For review of the LTB structure and assembly into pentameric form, see Sixma, et al., 1993, *J.Mol. Biol.* 230:890-918, "Refined structure of *Escherichia coli* heat-labile enterotoxin, a close relative of cholera toxin". The crystal structure and three dimensional coordinates of the B subunit are known (Sixma, et al., *Nature* 351:371-377, 1991).

In the B_5 pentamer, the identical B subunits interact with themselves, but also have loops that are exposed on the surface of the pentamer. Each subunit takes part in approximately 30 inter-subunit hydrogen bonds and six salt bridges with its two neighbors. Although a large portion of the B subunit's surface area is buried inside the structure of the AB_5 or B_5 complexes, several loop structures are exposed on the surface of the subunit, and/or on the surface of the associated pentameric complex, as shown by X-ray crystallography. The loops are parts of the secondary structure of the B subunit, which includes a small N-terminal helix, two three-stranded anti-parallel sheets, and a long alpha-helix. Loops in the LTB subunit provide connections between strands and are believed to provide shape to the molecule's binding cavities

Analysis of the LTB protein's structure [Seq. ID NO:2] by, for example, interactive computer graphic modeling, identified several domains of the pentamer appropriate for display of inserted epitopes. For example, appropriate insert regions include external loops formed at amino acid positions 10-15; 22-26; 30-37; 41-47; 50-61; 77-82; and 88-94. The sequence of the *etxB* gene, which encodes each of the identical B subunit proteins, is known [Seq. ID NO:1] (Yamamoto, et al., 1984, *J. Biol. Chem.*, 259:5037-5044) and the coding sequences for each of the loops can be determined. The nucleotide residues encoding the loops include positions 109-121; 203-218; 229-260; 310-323, and 343-359. One preferred loop sequence includes nucleic acid residues 229-260, and contains a unique restriction site, *SmaI* at nucleotide 237. Unique restriction sites can also be engineered into other exposed LTB loops by recombinant DNA technology, permitting ease of insertion of a desired antigen. For example, an external α-helix encoded by *etxB* nucleic acid residues 92-110 can be engineered to contain a unique *Bgl II* site at nucleotide 97, by replacing *etxB* nucleotide 100 with thymine.

Using these unique restriction sites, foreign nucleic acid sequences encoding small peptide antigens from a desired protein may be inserted into a the *etxB* to form a nucleic acid construct. The small peptide antigen can be a fragment of a larger protein. The foreign sequences encoding a desired antigenic sequence can be inserted so that the reading frame encoding LTB is not disrupted and the peptide antigen is expressed within the LTB sequence. The fusion proteins

containing antigenic peptide sequence and the LTB sequence are immunoreactive with antibodies that recognize or bind to the inserted peptide or to an epitope of a protein containing the sequence of the inserted peptide. The immunogenic fusion proteins, or chimeric LTB molecules, can be expressed by methods known for expressing proteins in host cells. On expression of the chimeric LTB molecule, the inserted peptide antigen is expressed on the surface of the molecule and presented for immune response in a host animal. The multimeric structure of LTB allows presentation of multiple antigens on the multiple exposed surfaces of a single pentamer.

Antigens

Antigenic peptides useful in the present invention are generally short amino acid sequences, e.g., approximately 8-30 amino acids, and preferably 10-25 amino acids in length. The peptide is preferably known to be unique to a specific protein of choice, and represents an epitope that is able to induce a desired immune response against the protein target. For example, the antigenic peptide may be known to produce a desired antigenic response when used in another carrier protein/adjuvant system such as co-administration with Fruend's Adjuvant or other immunogen. Alternatively, the peptide antigen may be a portion of a known protein having a particularly unique amino acid sequence distinguishing it from other proteins. These and other techniques for identifying and screening potential antigenic peptides useful in vaccine development are generally known. See, for example, Scott, et al., 1990, *Science* 249:386-390.

Antigenic peptides may be inserted into the LTB molecule by recombinant DNA methods. For example, a synthetic nucleic acid sequence or vector containing the desired nucleic acid sequence to be inserted into *etxB* is specifically designed to include restriction endonuclease sites matched to a specified endonuclease-cut *etxB* sequence. Where the *etxB* contains a single, unique restriction endonuclease site, the antigen's nucleic acid sequence preferably is engineered to include matched restriction sites at both ends of the sequence, so that it can be inserted into the *etxB* sequence without removal of any *etxB* nucleotides. Care is taken to match the antigenic nucleic acid sequence to be inserted with the

reading frame of the *etxB* sequence so that normal expression of the encoded LTB and the encoded antigen is achieved.

Insertion of the antigen's nucleic acid sequence and expression of the antigenic peptide does not interfere with normal expression of LTB monomers or with folding of the molecule. Preferably, insertion of the antigenic peptide does not interfere with the association of the LTB monomers into pentameric form. Most preferably, the inserted antigenic peptide does not interfere with LTB's three dimensional structure, and permits presentation and recognition of the inserted antigen on an exposed surface of the three-dimensional pentameric form of the molecule.

It is contemplated that the compositions and methods of the invention may be limited by the antigenic peptide's amino acid chain length (e.g., no greater than about 30 amino acids), net charge of the inserted amino acid sequence (e.g, less than about 50% highly charged amino acid residues), potentially cross-linking residues, or a density of potentially self-hybridizing nucleic acid sequences. These limitations are generally known and can be recognized by review of the amino acid sequence to be inserted.

It is generally known that a nucleic acid sequence may be modified for enhanced expression in a particular host cell by modifying the codons of the nucleic acid sequence to those more preferred in the specific host cell. Thus, for example, to express the LTB-antigen in *E. coli*, the peptide sequence may be back translated into the nucleotide sequence using the codon frequency found in *E. coli* proteins, as determined by the GCG computer program (Devereaux, et al., 1984, *Nucleic Acids Res.* 12:387-3905) modified as suggested by *E. coli* codon frequencies.

It is generally understood that protein expression in a given host cell may be enhanced by modification of one or more nucleotides in the coding sequence to reduce the number of unique or rare codons. In a preferred embodiment of the invention, the nucleic acid sequence contains one or more codons modified according to the condon frequency preferences for a particular cellular host.

Inhibin Vaccine

Inhibin is a glycoprotein produced by the gonads that selectively suppresses the secretion of follicle stimulating hormone (FSH) from the anterior pituitary gland. A vaccine against inhibin can decrease available inhibin, with a resulting increase in levels of follicle stimulating hormone, and enhanced fertility. Enhanced fertility can be due to enhanced production of sperm or ova. Immunization of animals with bovine inhibin - αC subunit has demonstrated the usefulness of inhibin-based antigens as fertility-enhancing vaccines. However, to date, a practical commercial vaccine has not been produced, at least in part due to the limitations of chemical synthesis, conjugation, and adjuvant toxicity discussed above.

In a most preferred embodiment and exemplary of the invention, the nucleic acid sequence encoding the first 14 N-terminal residues of the antigenic inhibin α_c subunit (αC^{1-14}) is inserted into the unique *SmaI* restriction site of *etxB*. Alternatively, the nucleic acid sequence encoding the first 26 N-terminal residues (αC^{1-26}) is inserted. The chimeric gene is subcloned into a broad-host-range expression vector. The inserted antigen is expressed on the surface of the LTB molecule, such that when the expressed chimeric protein vaccine is injected into host animals, an anti-inhibin response is induced in the animals, reducing inhibin and thereby enhancing fertility in treated animals.

Cellular Hosts

Many known cellular host systems are suitable for expression of the chimeric genes of the invention. For example, non-pathogenic strains such as *Vibrio* and including *Vibrio anguillarium* are transfected with suitable vectors containing the gene encoding LTB-antigen and express the fusion protein. Suitable vectors for use in *Vibrio* include pJF118, as described in Furst, et al, 1986, *Gene* 48:119-131.

Methods of Administration

LTB is a known immunogen. The immunogen of the invention, formed of the intact LTB protein and an inserted antigenic peptide, are administered

according to the methods known as effective for the immunogenic administration of proteins such as LTB.

Administration methods include injection of protein compositions to induce effective antibody titers. In a preferred embodiment, the fusion protein of the invention is expressed in edible plants or animals for oral ingestion. This oral delivery method has been described for immunogenic delivery of LTB. See, for example, Mason et.al., 1995, *TIBTECH* 13:388-392, describing oral immunization against LTB via ingestion of transgenic potato tubers expressing LTB antigen.

Examples

The present invention may be better understood with reference to the following examples. These examples are intended to be representative of specific embodiments of the invention, and are not intended as limiting the scope of the invention.

Example 1

Analysis of the 3-D Structure of LTB

The B subunit of the *E.Coli* heat-labile enterotoxin (LTB) is a multimeric protein composed of five identical polypeptides of about 11 kDa each. The three-dimensional crystal structure of LTB was analyzed using interactive computer graphic modeling for potential exposed, antigenic regions appropriate for the display of inserted epitopes. Specifically, the structure was analyzed to identify potential sites for insertion of small peptides for purposes of producing potentially antigenic molecules or vaccines.

The protein's structure was examiner with the Biosym modelings program (Biosym Technologies, 1985, Scranton Road, San Diego, CA). Domains of the LTB pentamer that are exposed on the surface of the molecule were identified by examination of stereo images. The domains encoding external loops useful for insertion of antigens were selected using the sequence analysis program GCG (Devereaux, et al., 1984, *Nucleic Acids Res.* 12:387-3905). Possible insertion sites were identified as loops in the three-dimensional structure that could potentially

tolerate additional amino acids. These loops were also exposed on the surface of the molecule, indicating potential antigen presentation. Aligning the amino acid sequence and then nucleic acid sequence with the identified structural site, potentially useful insertion sites were found.

A potential insertion site is positioned approximately at nucleotide 237, and contains a unique *Sma*I endonuclease restriction site in the native sequence. A second potential insertion site is positioned at approximately nucleotide 97. While this second site does not contain a unique restriction site in the native sequence, one nucleotide was modified by site-directed mutagenesis to make this site available for direction insertion of foreign oligonucleotides. By replacing adenine at position 100 with thymine, a unique *Bgl* II restriction site was created at nucleotide 97. In the modified protein encoded by the mutant *etxB*, Glu-7 is replaced by Asp as shown below:

Wild Type *etxB*

(Seq. ID NO:3)

```
   GCTCCTCAGTCTATTACAGAACTATGTTCGGAATATCAC
80 +---------+---------+---------+-------- 118
   CGAGGAGTCAGATAATGTCTTGATACAAGCCTTATAGTG
 1 AlaProGlnSerIleThrGluLeuCysSerGluTyrHis  13
```

(Seq. ID NO:4)

Mutant E7D0*etxB*

(Seq. ID NO:5)

```
   GCTCCTCAGTCTATTACAGATCTATGTTCGGAATATCAC
80 +---------+---------+---------+-------- 118
   CGAGGAGTCAGATAATGTCTAGATACAAGCCTTATAGTG
 1 AlaProGlnSerIleThrAspLeuCysSerGluTyrHis  13
```

(Seq. ID NO:6)

A third potential insertion site was created at nucleotide 176 by substitution of the two adenine nucleotides at positions 179 and 180 with cytosine and thymine nucleotides, respectively. This mutation created a unique *Stu*I site at nucleotides 176-180. This mutation also created a substitution of the Lys-34 by Leu

residue. The resulting mutant LTB subunit was expressed and formed pentamers as did the wild type LTB.

Wild Type *etxB*

(Seq. ID NO:7)

```
      ATGGCAGGCAAAAGAGAAATGGTTATCATTA
170 +---------+---------+---------+ 200
      TACCGTCCGTTTTCTCTTTACCAATAGTAAT
 31 MetAlaGlyLysArgGluMetValIleIle   40
```

(Seq. ID NO:8)

Mutant K43L-*etxB*

(Seq. ID NO:9)

```
      ATGGCAGGCCTAAGAGAAATGGTTATCATTA
170 +---------+---------+---------+ 200
      TACCGTCCGGATTCTCTTTACCAATAGTAAT
 31 MetAlaGlyLeuArgGluMetValIleIle   40
```

(Seq. ID NO:10)

To create these sequences, wild type LTB was constructed as described in (Sandkvist, et al., 1987, *J. Bacteriol.* 169:4570-4576). Site-directed mutagenesis was performed by the method of Eckstein (Sayers, et al., 1988, *Nucleic Acids Res.* 16:791-802). The sequences of the mutant genes obtained were verified by nucleotide sequence determination by di-deoxy sequencing procedures (Sanger, et al., 1977, *Proc. Nat'l. Acad. Sci. USA*, 74:5463-5467).

Example 2

Production of a LTB-Inhibin Fusion Polypeptide

A nucleic acid sequence encoding an immuno-dominant epitope of inhibin, was inserted into the nucleic acid sequence encoding LTB (*etx*B) at its unique *Sma*I site. To accomplish the insertion of the sequence, the N-terminal portion of the αC-subunit was back-translated into the nucleotide sequence using the codon frequencies found in *E. coli* proteins,using the GCG program as described

above (Devereaux, et al., 1984, *Nucleic Acids Res.* 12:387-3905). Complementary oligonucleotides of this back-translated sequence were chemically synthesized (MSU Macromolecular Structure Facility, Biochem. Dept.) and inserted into *etxB* in a manner that ensured the continuation of the reading frame after insertion at the *Sma*I restriction site, as shown in the sequences in the table below.

The pair of complementary oligonucleotides (Seq.ID No: 11) encoding bINHαC$^{1-14}$ (Seq. ID No:12) was inserted into the pMMB68 vector (Sandkvist, et al., 1987, *J. Bacteriol.* 169:4570-4576), a broad host-range vector, containing the *etxB* sequence under the control of the inducible isopropyl - β-D thiogalactopyranoside (IPTG) *tac* promoter (Sandkvist, et al., 1987, *J. Bacteriol.* 169:4570-4576), digested with *Sma*I, as shown below. The sequences of the insert are shown in bold type, the nucleotide numbers of the *etxB* sequences are indicated, and extra nucleotides introduced to maintain the proper reading frame, are underlined. The encoded amino acid sequence is also shown.

230 240
GTCGAAGTCCCTGGATCCACCCCGCCGCTGCCGTGGCCGTGGTCCCCGGCTGCTCTGGGCAGTCAA
(Seq. ID No:11)
CAGCTTCAGGGACCTAGGTGGGGCGGCGACGGCACCGGCACCAGGGGCCGACGAGACCCGTCAGTT
ValGluValProGlySerThrProProLeuProTrpProTrpSerProAlaAlaLeuGlySerGln
(Seq. ID No:12)

Recombinant plasmids containing the inhibin subunit sequence were introduced into *E. coli* cells (CB1360, GIBCO BRL, Life Technologies) by standard calcium phosphate transformation methods. A diagram showing the constructed plasmid (pMMB552) is shown in Figure 1.

Colonies of transformed *E.Coli* were screened for inhibin and LTB expression by immunoblotting. Colonies were grown on parallel nitrocellulose membrane filters (Schleicher & Schuell), placed on nutrient agar (LB) plates containing ampicillin and IPTG as inducer.

A first filter was blotted with anti-inhibin antibody, and a second filter was blotted with anti-LTB antibodies. Whole cell inhibin immunoblot assays using mink anti-bINHαC$^{1-26}$ gly·tyr antiserum (Ireland, et al., 1994, *Biol. Reprod.*

50:1265-1276) or anti-pentameric LTB monoclonal antibody 118-8 (Sandkvist, et al., 1987, *J. Bacteriol.*, 169:4570-4576) were performed separately on each of the two parallel membranes. Colonies exhibiting both inhibin and LTB immunoreactivity were selected for further DNA sequence screening. DNA from these colonies was isolated by standard methods, cloned into the sequencing vector, M13mp19 and sequenced. The sequence analysis of both DNA strands was done by automated fluorescent sequencing (MSU-DOE-PRL Plant Biochemistry Facility) using ABI catalyst 800 Taq cycle sequencing and the ABI 373A sequencer for the analysis of products. One of the many colonies containing the expected *etx*B:bINHα_c^{1-14} DNA sequence wasidentified as harboring pMMB552 and was retained for continued analysis and development.

To determine if the fusion *etxB*-inhibin protein exhibited the same pentameric subunit structure as wild type B-subunit, protein produced by *E. coli* cells containing pMMB522 was run on SDS-polyacrylamide gels without denaturation (no boiling of samples) and with denaturation (boiling for 5 minutes).

The electrophoretic mobility of the non-denatured fusion protein was about the same as that of the native LTB. After denaturation, the pentameric form dissociated into monomeric subunits that ran slightly slower than the wild type-B monomers, reflecting their larger size.

Example 3

Expression and Secretion of LTB-Inhibin Fusion Polypeptide in Cellular Host Systems

The LTB-Inhibin fusion construct expressing the LTB-inhibin fusion protein, prepared as described for Example 2, was introduced into *Vibrio cholerae* TRH (Sandkvist, et al., 1987, *J. Bacteriol.* 169:4570-4576) and *Vibrio anguilarum* H3 (Crosa, 1980, *Infec. Immunity,* 27:897-902) by conjugative mobilization under conditions sufficient to produce chimeric protein at concentrations greater than 6 mg/l. Conjugation was achieved by making a mixed suspension of the donor strain (*E. coli* CB1360 harboring pMMB552), the helper strain, HB101pRK2013:kanamycin resistant (Km^R), and the recipient strains (*V. anguillarium* Rif^R or *V. cholerae* TRH 7000: polymyxin resistant, Pmx^R) at a 1:1:1

ratio in LB broth in a sterile 1.5 ml eppendorf tube. The suspension mixture was plated on LB medium as a discrete droplet and incubated overnight at 37°C.

The overnight bacterial growth was scraped off the plate and suspended in sterile 0.9% saline solution, then replated on a Rif, A_p selective plate. Selected colonies were individually streaked on fresh LB media (1.5% W/V Bacto-agar plates containing Luria Broth (LB, Difco, Detroit, MI) supplemented with 1.5% NaCl and 100 μg/ml ampicillin (Ap, Sigma, St. Louis, MO). Plates were incubated overnight at 30°C. A single colony was picked from the plate, grown in 20 ml LB, and inoculated into one liter LB supplemented with 1.5% NaCl and 100 μg/ml Ap for culture to an absorbance at 650 nm of 0.02-0.05. At $A_{650} = 0.02$, IPTG was added to a final concentration of 1.0 mM to induce transcription of *etx*B. Cultures were harvested 6 or 48 hours after IPTG addition, and medium was saved for analysis and protein purification.

A one ml sample of the cell culture was separated by centrifugation. The separated cells were resuspended in 1.0 ml PBS and broken by sonication with two ten second pulses. The amount of LTB pentamers present in the growth medium and in the lysed cells was determined by GMI ELISA using the methods described in Svennerholm and Holmgren, 1978, *Curr. Microbiol.*, 1:19-27. Briefly, pentameric, but not monomeric LTB binds to galactosyl-N-acetogalactosaminyl-(N-acetylneuraminyl)galactosylglucosylceramide (GM1). To determine if the expressed fusion protein, *etxB*:bINHαC$^{1\text{-}14}$, retained the LTB pentameric structure, the produced protein was tested for its ability to bind ELISA plates coated with GM1 ganglioside (Sigma). Wild type LTB was used as control. The amount of LTB in each sample was estimated from the ED_{50} of the *E.coli* enterotoxin standard curve. The percent LTB in the media was calculated as the amount in the media/the amount in media plus cells x 100%.

Table 1.

Bacterial Strains	*EtxB* (μg)		*%EtxB* Pentamers in Media
	Cells	Media	
***EtxB*:bINHα$_c^{1-14}$**			
V.cholerae	148.8	16.0	9.0
V.anguillarum	92.2	91.6	50.0
EtxB			
V.cholerae	0.0	57.0	100.0
V.anguillarum	4.6	70.0	94.0

As shown in Table 1 above, both host cells *V. anguillarum* and *V.cholerae* produced the recombinant fusion protein. Although the total amount of fusion protein produced in each host was similar, more fusion protein was detected in the medium of *V.anguillarum* as compared with *V.cholerae*.

Fusion protein accumulated in the medium of 1 liter cultures was precipitated with ammonium sulfate (65% saturation) according to the methods described in Amin et.al., 1993, *Biochem. Soc. Trans.*, 21:213S. The precipitate was recovered by centrifugation (10,000 x g, Beckman L-80 ultracentrifuge) for 25 minutes, was redissolved in PBS containing 5% glycerol, and ws dialized against the same buffer at 4°C.

An ion exchange column (CL 6B DEAE-Sepharose, 9.5 x 1.6 cm, Pharmacia, Piscataway, NJ) equilibrated with PBS containing 5% glycerol, was used to further purify the fusion protein. An increasing NaCl gradient (137 to 600 mM in PBS-glycerol) was used to elute fractions. An aliquot of each collected fraction was diluted 1:1000 in PBS-Tween containing 0.1% BSA and assayed by GM1 ELISA as described above to detect pentameric fusion protein. Fractions with the highest amount of the fusion protein were pooled, and the protein mass estimated from spectrophotometer readings using the Warburg equation: $A_{280} = 1$ for 0.1% protein solution. LTB specific activity was estimated as the amount of GM1-reactivity as determined by the GM1 assay per total protein estimated by spectrophotometer readings.

The fusion protein was eluted as a single peak between 300 and 430 mM of the NaCl gradient. An average of 84.9 +/- 21.5 mg of the fusion protein was isolated (67 +/- 11% recovery) with an LTB specific activity of 0.84 +/- 0.02 per liter.

Protein eluted from the ion exchange column was assessed for purity and molecular weight by SDS-PAGE. Pentameric or heat-disrupted (100°C) monomeric samples (1.2 to 3.6 µg) of LTB or the fusion protein, LTB:bINHα_C^{1-14} were separated in one dimensional 12% SDS-PAGE in a mini-gel apparatus following the manufacturer's instructions (Mini-Protean II, BioRad). Separated proteins were visualized by Coomassie blue staining and compared with molecular weight markers for estimation of size.

A single major band for each of the pentameric LTB (39.5 ± 1 kDa) and monomeric LTB (8.9 ± 0.4 kDa) was observed in the stained gels. The major band for each of the pentameric LTB:bINHα_C^{1-14} (41.6 ± 2 kDa) and monomeric LTB:bINHα_C^{1-14} (10.1 ± 0.2 kDa) were larger than the LTB bands, according to the expected size of the insert.

The pentameric or heat-disrupted monomeric fusion protein samples were further analyzed for inhibin and LTB specificity by immunoblotting techniques. Briefly, duplicate samples were electrophoresed in 12% SDS-PAGE and electroeluted onto nitrocellulose membranes, as described above. After blocking with 0.01% Blotto (Food Club, Skokie, IL) in TBS for 2 hours, the membrane was washed in Tween-TBS (0.05% Tween-20), and cut to present two replicate membranes for antibody binding.

One of the membranes was incubated with mink anti- bINHα_C^{1-26}gly.tyr antiserum (1:1000 in TTBS) overnight at room temperature, as described in Ireland et.al., 1994, *Biol. Reprod.*, 50:1265-1276. After washing in TTBS (5, 10-minute washes), the membrane was further incubated in 20 ml ^{125}I- bINHα_C^{1-26}gly.tyr (1 x 10^6 cpm/ml in TTBS with 1% gelatin), for competition. The membrane was washed and placed on Xray film (Kodak X-OMAT AR) with a Cronex intensifying screen and exposed for ten days at -80°C.

The second membrane was incubated with a monoclonal anti-LTB antibody (118-8, 1:100 dilution) as described in Sandvikst et.al., 1987, *J. Bacteriol.*,

169:4570-4576. Incubation with second antibody goat anti-mouse peroxidase conjugate (Vector) diluted 1:5000 in TTBS was followed by visualization in 0.2% (w/v) 4-chloro-1-napthol (Sigma) in PBS containing 20% methanol and 0.01% H_2O_2.

As shown in Figure 2, the immunoblot assays confirmed LTB:bINHα_C^{1-14} fusion protein was produced having dual inhibin and LTB immunoreactivity. Anti-LTB antibody recognized both wild type LTB and the fusion protein in both monomeric and pentameric forms. Anti-bINHα_C^{1-26}gly.tyr antiserum recognized only the monomeric and pentameric forms of the fusion protein, and did not bind the LTB alone. Molecular weight determinations for the immunoreactive proteins were similar for the monomeric (11kDa, open arrow) and pentameric (45 kDa, shaded arrow) forms as compared with the size estimates obtained from Coomassie blue stained gels.

Inhibin Radioimmunoassay (RIA)

Inhibin immunoactivity in duplicate samples of the fusion protein (0.5-64 μg) in both monomeric and pentameric forms, and in control samples, including bINHα_c^{1-26} gly.tyr peptide standards (0.039 ng - 10 ng), pentameric and monomeric LTB (0.625μg - 80μg), was determined by radioimmunoassay using mink anti-bINHα_c^{1-26}gly.tyr antiserum diluted 1:40,000 in RIA buffer (0.01M NaH_xPO_4, 0.1M NaCl, 0.025M EDTA, 0.1% NaN_3, 0.1% Triton X-100, 0.1%BSA). ^{125}I-bINHα_c^{1-26}gly.tyr (20,000 cpm/tube) was used as tracer. The RIA conditions were as described in Ireland, et al., 1992 *Biol. Reprod.*, 47:746-50; and Good, et al., 1995, *Biol. Reprod.*, 53:1478-1488. Briefly, 200 μl of protein sample, 200 μl mink anti-bINHα_c^{1-26}gly.tyr antiserum and 100 μl ^{125}I-bINHα_c^{1-26}gly.tyr tracer were sequentially added into test tubes and incubated for 16-18 hours at 4°C. The antibody: ^{125}I-bINHα_c^{1-26}gly.tyr complex was incubated for 2 hours with 100 μl *Staphylococcus* protein A (Staph A; Boehringer Mannheim) diluted 1:50 in RIA buffer at room temperature, followed by the addition of 2 ml/tube PBS (pH 7.4 with 0.025 M EDTA) and centrifugation for 30 minutes at 2,200 x g at 4°C (Beckman GPR centrifuge) to sediment the inhibin-antibody complex. Tubes were decanted and radioactivity in the Staph A:antibody:^{125}I-bINHα_c^{1-26}gly.tyr complex determined

using a MACC Micromedic γ-counter. Inhibin immunoactivity was plotted as percent ^{125}I-bINHα_c^{1-26}gly.tyr tracer bound.

As shown in Figure 3, the pentameric fusion protein, LTB:bINHα_c^{1-14} reacted with the anti-inhibin antiserum parallel to the reaction of bINHα_c^{1-26}gly.tyr peptide. In a separate RIA, monomeric fusion protein did not react with the antiserum (Figure 4).

Example 4

Passive Immunization of Mice and Rabbits with Anti-EtxB:bINHα_c^{1-14} Antiserum

Use of the LTB:bINHα_c^{1-14} fusion protein as a fertility vaccine requires that LTB:bINHα_c^{1-14} stimulates production of serum inhibin antibodies when injected into animals, ideally in the absence of adjuvant. As shown above in Example 3, pentameric form of LTB:bINHα_c^{1-14} fusion protein cross-reacted in a parallel fashion with a synthetic bINHα_c^{1-26} gly.tyr. peptide during radioimmunoassay (RIA) indicating that bINHα_c^{1-14} peptide is on the hydrophilic surface of the intact, non-denatured molecule. These RIA data imply that the bINHα_c^{1-14} peptide portion of EtxB:bINHα_c^{1-14} is immunogenic, especially since immunogenicity is closely correlated with hydrophilicity (Sagar, et al., 1989 *J. Pept. Protein Res.* 33:452-456). In addition, bINHα_c^{1-14} peptide was inserted between amino acid 53 and 54 in EtxB a region of LTB known to be highly immunodominant (Jacob, et al., 1984 *EMBO J.* 3:2889-2893, and 1985 *EMBO J.* 4:3339-3343).

Animals

Rabbits and mice were maintained by the University Laboratory Animal Resources (ULAR, Michigan State University) in their animal care facilities for the duration of the experiments. All animals were maintained on a 12L:12D cycle with food and water provided *ad libitum*.

Treatments

I. Active Immunization

Adult female New Zealand White rabbits of 6-7 kg body weight were purchased from ULAR and housed 1/cage. Purified LTB:bINHα_c^{1-14} fusion protein produced in *Vibrio anguillarum* as described in Example 3, was mixed with or without Freund's adjuvant and used to actively immunize rabbits. For antigen preparation in Freund's adjuvant, 3 ml LTB:bINHα_c^{1-14} in phosphate buffered saline (PBS - 0.01M phosphate buffer, pH 7.4 with 0.15M NaCl) was mixed with an equal volume of Freund's complete (primary injection) or incomplete (boosters) adjuvants (Calbiochem, La Jolla, CA) to give a final sample concentration of 100 (primary) or 50 (boosters) μg LTB:bINHα_c^{1-14} per ml. Antigen and adjuvant were mixed in 12 x 75 mm glass test-tubes and emulsified using a 10 ml syringe with an 18 gauge needle. Mixing was done by suction and expulsion of the antigen and adjuvant through the needle until a stable emulsion was obtained. The emulsion was considered stable when a droplet of emulsion on the surface of the water in a beaker did not disperse when the beaker was shaken. For antigen preparation without Freund's adjuvant, LTB:bINHα_c^{1-14} fusion protein was diluted in PBS to a concentration of 100 (primary) or 10 (boosters) μg LTB:bINHα_c^{1-14} per 0.5 ml (2 ml total volume) and sterilized by expulsion through a 2.2 μm Millex GV sterile filter (Millipore, Bedford, MA) attached to a 5 ml syringe into a 15 ml sterile polypropylene tube.

All rabbits were bled from the marginal ear vein before immunization to recover preimmune serum which was used as the negative control. For the adjuvant group (n=3), the primary dose (volume = 1 ml) of LTD:bINHα_c^{1-14} was given s.c. in the nape of the neck or in the back at 10 sites (0.1 ml per site) followed by five s.c. boosters at 2-week intervals. For the group immunized without Freund's adjuvant (n=3), the primary dose of LTB:bINHα_c^{1-14} (volume = 0.5 ml) was injected i.v. into the marginal ear vein followed by five boosters at 2-week intervals. LTB:bINH injections were performed while restraining rabbits in a towel (ULAR recommendations). To collect blood, rabbits were anesthetized with sodium phenobarbitone (Nembutal, Sigma, St. Louis, MO) and blood was removed from a marginal ear vein (5-15 ml) or the heart (100-150 ml) using a 20 or 50 ml syringe

with a 20 gauge needle. Serum samples were collected 14 days after each booster for a total of five bleeds per rabbit. Blood samples were incubated at room temperature for 2 hours and then at 4°C overnight. Serum was separated from clotted blood cells by centrifugation at 1000 x g for 30 minutes at 4°C then stored at -20°C until assayed by ELISA. Serum from three rabbits with the highest inhibin antibody titer was pooled and used to passively immunize mice.

II. Passive Immunization

Twenty prepubertal male BALB/c mice (25 days old) averaging 12.2 ± 1.4 grams body weight were purchased from Harland Sprague Dawley (Wilmington, MA) and housed 5/cage. Rabbit anti-LTB:bINHα_c^{1-14} antiserum generated, as described above, was used to passively immunize mice, and preimmune serum was used as control. Crude anti-LTB:bINHα_c^{1-14} antiserum or preimmune serum was filter-sterilized through a 2.2 μm Millex GV sterile filter attached to a 5 ml syringe and collected into 15 ml sterile polypropylene tubes.

Mice were divided into four groups (5 mice/group). The first two groups were given one 0.5 ml i.p. injection of either rabbit anti-LTB:bINHα_c^{1-14} antiserum or preimmune control serum, whereas the other two groups received two, 0.5 ml i.p. injections of either anti-LTB:bINHα_c^{1-14} or preimmune serum spaced 12 hours apart. After mice were anesthetized with Metofane (Methoxyflurane; Pitman-Moore, Mundelein, IL), blood was collected via heart puncture 12 hours after the last injection in each group, serum processed, as described above in Active Immunization, and inhibin antibody titer and concentrations of FSH and LH in serum determined, as described below.

Antibody Titer Determination by ELISA

I. Anti-LTB Antibody Titer

A modification of the GM1 ELISA, described in Svennerholm and Holmgren, 1978, *supra*, was used to determine anti-LTB antibody titer in serum from actively immunized rabbits (5 bleeds/rabbit). Probind microtiter plates (Falcon, Lincoln Park, NJ) were coated overnight at room temperature with 0.2 μg/well galactosyl-N-acetogalactosaminyl-(N-acetylneuraminyl)-galactosyl

glucosylceramide (GM1) in PBS. After washing wells three times with PBS containing 0.05% Tween-20 (PBS-T), non-specific binding sites were blocked by adding 1% BSA (Sigma) in PBS-T to each microwell and incubating for 2 hours at room temperature. Plates were washed and incubated with 100 ng/well partially purified pentameric LTB for 1 hour at room temperature. After another wash, microwells were incubated with serum from actively immunized rabbits diluted 1:5000 in PBS-T containing 0.1% BSA (PBS-T-B) for 1 hour at room temperature. After washing with PBS-T, horseradish peroxidase-labeled goat anti-rabbit IgG diluted 1:5000 (Vector, Burlingame, CA) in PBS-T-B was added to each microwell and incubated for 1 hour at room temperature. Color was developed using ortho-phenylenediamine (OPD) in 0.1M citrate buffer, pH 4.5, containing 0.01% H_2O_2. After 10 minutes, color development was terminated by adding 3M phosphoric acid. Titer of anti-LTB antibodies in serum was defined as absorbance at 490 nm (A_{490}, Bio-Rad microplate reader, Model 3550).

II. Inhibin Antibody Titer

A modified ELISA method, as described in Groome and O'Brien, 1993 *J. Immun. Methods* 165:167-176, was used to estimate titer of anti-inhibin antibodies in serum. Xenobind microtiter plates (Xenopore Inc., Hawthrone, NJ) were covalently coated overnight at room temperature with 1μg/well bINHα_c^{1-26} gly.tyr. peptide or 1 μg/well partially purified bovine inhibin ppbINH, prepared as described in Good, et al., 1995, *Biol. Reprod.*, 53:1478-1488, in PBS as recommended by the manufacturer. After washing wells three times using PBS-T, non-specific binding sites were blocked by incubating wells with 3% gelatin (Bio-Rad) in PBS-T for 2 hours at room temperature. Plates were washed and the coated microwells incubated for 2 hours with serum from actively immunized rabbits or passively immunized mice diluted 1:100 in PBS-T-B. After washing with PBS-T, horseradish peroxidase labeled goat anti-rabbit IgG (Vector, Burlingame, CA) diluted 1:5000 in PBS-T-B was added to each microwell and incubated for 1 hour at room temperature. Microwells were thoroughly washed, color was developed using OPD-H_2O_2, and titer of inhibin antibodies in serum was determined, as described in LTB antibody titer.

FSH and LH RIA

I. Iodination and Validation

Concentrations of FSH and LH in serum from passively immunized mice were determined in duplicate samples using rat FSH and LH reagents kindly supplied by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK). The chloramine-T method described in Hunter and Greenwood, 1962, *Nature* 194:495-496, was used to radiolabel 5 μg of rFSH (NIDDK-rFSH-I-8) or rLH (NIDDK-rLH-I-9) dissolved in 20 μl PBS in a Nalgene cryovial (Nalge, Rochester, NY). Iodinated rFSH and rLH was stored at 4°C for use as tracer in each RIA.

The ability of radioiodinated hormone to bind antiserum was determined in duplicate by incubating 50 μl antiserum (NIDDK-anti-rFSH-S-11 or NIDDK-anti-rLH-S-11) diluted 1:1000 to 1:640,000 in assay buffer (0.0095 M Na_2HPO_4, 0.014 M NaH_2PO_4, 0.15 M NaCl, 0.01 M EDTA, 0.1% NaN_3, 0.5% Chicken Egg Albumin, pH 7.2) with 100 μl assay buffer and 50 μl ^{125}IrFSH or ^{125}IrLH tracer diluted to 12,000 cpm/tube with assay buffer. Tubes were incubated in the aforementioned buffers for 18 hours at room temperature followed by: 1) precipitation of the bound antibody by incubating tubes with 50 μl/tube of *Staphylococcus* protein A (Staph A; Boehringer Mannheim) diluted 1:100 in PBS-EDTA (PBS, pH 7.4, 0.025 M EDTA) for 1.5 hours at room temperature; 2) addition of 2 ml/tube PBS-EDTA to wash tubes; and 3) immediate centrifugation for 30 minutes at 2,200 x g at 4°C (Beckman GPR centrifuge) to sediment the Staph A:antibody:tracer complex. The tubes were decanted and radioactivity in the dried pellet determined in a MACC Micromedic γ-counter. Percent binding of tracer to antibody was calculated as:

$$\% \text{ binding} = \frac{\text{(average cpm for each dilution)}}{\text{average total cpm}} \times 100$$

An antiserum dilution of 1:100,000 resulted in 20% binding of tracer to LH or FSH antibody, thus the 1:100,000 dilution was used in RIA of rFSH or rLH in serum (data not shown).

II. RIA

Mouse FSH and LH cross-react with the NIDDK rat gonadotropin antibodies (Beamer, et al., 1972 *Endocrinology* 90:823-827; Kovacic and Parlow, 1972 *Endocrinology* 91:910-915). In the present study, several dilutions of BALB/c mouse serum were used to confirm parallelism of mouse serum to the standard curve produced by NIDDK-rat-FSH-RP-2 or NIDDK-rat-LH-RP-3 reference preparations. The standard FSH and LH assay (Parkening, et al., 1980) was miniaturized to reduce the total incubation volume from 600 µl to 200 µl. Duplicate mouse serum samples (5 to 50 µl) diluted to 100 µl in assay buffer were incubated with 50 µl antiserum (1:100,000 in assay buffer, NIDDK-anti-rFSH-S-11 or NIDDK-anti-rLH-S-11) at room temperature for 18 hours. The following day, 50 µl tracer was added at 12,000 cpm/tube and the mixture further incubated at room temperature for 24 hours. This second incubation was followed by precipitation with Staph A as described in Iodination and Validation. Tubes were decanted and the radioactivity in each dried pellet determined in a MACC Micromedic γ-counter. FSH values were expressed in terms of the rat FSH-NIDDK-RP-2 reference standard, whereas LH values were expressed in terms of the rat LH-NIDDK-RP-3 reference standard. Samples were analyzed in a single assay for each hormone. rFSH and rLH assay sensitivities were 0.625 and 0.156 ng/ml and intra-assay coefficients of variation (cv) were 6.3 and 1.6%, respectively. The cross reaction of FSH with LH and of LH with FSH was <2% (per NIDDK guidelines).

Statistics

Results were subjected to ANOVA. Whether significant ($P<0.05$) differences existed between means was determined by Student's *t*-test.

RESULTS

Active Immunization of Rabbits

Antibodies were generated to both LTB and bINHα_c^{1-14} components of the LTB:bINHα_c^{1-14} fusion protein in the two groups of rabbits injected with LTB:bINHα_c^{1-14} mixed with or without Freund's adjuvant. Specifically, both LTB and inhibin antibody titers reached a peak after the first booster and stayed elevated

for the duration of the experiment, although a transient decrease ($P<0.05$) in the inhibin antibody titer after booster 3 was observed (Figure 5). In addition, rabbits immunized with LTB:bINHα_c^{1-14} in Freund's adjuvant had anti-LTB antibody titers twice ($P<0.05$) as high as those immunized without Freund's adjuvant. However, anti-inhibin antibody titers were similar between rabbits injected with LTB:bINHα_c^{1-14} mixed with or without Freund's adjuvant.

As shown in Figure 5, preimmune serum did not bind to LTB and bINHα_c^{1-26} gly.tyr. peptide in ELISA. Antibodies generated against the LTB:bINHα_c^{1-14} fusion protein mixed with or without Freund's adjuvant bound to native inhibin but not to preimmune control serum.

Passive Immunization of Mice

I. Inhibin Antibody Titer Following Passive Immunization

The inhibin antibody titer was higher ($P<0.05$) in mice that received one injection of anti-LTB:bINHα_c^{1-14} antiserum compared with preimmune controls (Fig. 6A). Inhibin antibody titer was also higher ($P<0.05$) in mice that received two injections of anti-LTB:bINHα_c^{1-14} antiserum than those that received a single anti-LTD:bINHα_c^{1-14} injection or preimmune controls (Fig. 6A).

II. Serum Concentrations of FSH and LH

Administration of two injections of anti-LTB:bINHα_c^{1-14} antiserum resulted in a nearly two-fold increase ($P<0.05$) in plasma concentrations of FSH compared with preimmune controls (Fig. 6B). In contrast, concentrations of serum LH were similar ($P>0.1$) for anti-LTB:bINHα_c^{1-14}-treated and preimmune control mice (Fig. 6C).

The results of this study demonstrate that:

1) Immunization with LTB:bINHα_c^{1-14} fusion protein stimulated production of anti-bINHα_c^{1-14} antibodies when injected into rabbits with or without Freund's adjuvant;

2) Anti-bINHα_c^{1-14} antibodies stimulated by immunization with LTB:bINHα_c^{1-14} bound to native inhibin; and,

3) Anti-bINHα_c^{1-14} antibodies stimulated by immunization with LTB:bINHα_c^{1-14} effectively neutralized endogenous inhibin in host animals.

Example 5

Active Immunization of Mice with EtxB:bINHα_c^{1-14}

Animals

Mice were maintained by the University Laboratory Animal Resources (ULAR, Michigan State University) in their animal care facilities for the duration of the experiment. Male BALB/c mice (25 days old) were purchased from Harland Sprague Dawley (Wilmington, MA) and housed 5/cage, maintained on a 12L:12D cycle, and provided with food and water *ad libitum*.

Immunization Protocol

After Metofane anesthesia (Methoxyflurane; Pitman-Moore, Mundelein, IL), mice were bled by gently inserting a heparinized microhematocrit capillary tube (200 µl, Fisher, Pittsburg, PA) into the orbital sinus of the mouse. After filling the microhematocrit capillary tube with blood, the tube was sealed using hemato-seal tube-sealing compound (Fisher, Pittsburg, PA) then centrifuged for 5 minutes at full speed to recover preimmune plasma.

Purified LTB:bINHα_c^{1-14} and LTB proteins were used to actively immunize mice. Beginning at 26 days of age, male mice (n=5/group) were injected subcutaneously (s.c.) over a 10-week period at either 2- or 4-week intervals with two doses (10 or 40 µg) of LTB:bINHα_c^{1-14} fusion protein or one dose (40 µg) wild type LTB (controls). Each immunogen was injected in its pentameric or heat-disrupted monomeric form mixed with or without Freund's adjuvant. A group of ten untreated mice served as additional controls. To prepare the monomeric form, 500 µl aliquots of LTB:bINHα_c^{1-14} or LTB were heated in a heating block (100°C for 15 minutes) then left to cool at room temperature for 15 minutes before preparation for immunization. Henceforth, sample preparations for pentameric and monomeric LTB:bINHα_c^{1-14} and EtxB are similar.

For antigen preparation in Freund's adjuvant, 400 or 800 µl LTB:bINHα_c^{1-14} or LTB in PBS (0.01M phosphate buffer, pH 7.4 containing 0.15 M NaCl) was mixed with an equal volume of Freund's complete (primary injection) or incomplete (boosters) adjuvant (Calbiochem, La Jolla, CA) to give a final sample concentration of 10 or 40 µg LTB:bINHα_c^{1-14} or 40 µg LTB per 100 µl PBS. Antigen and adjuvant were mixed to give a stable emulsion as described for Example 3. For preparation of LTB:bINHα_c^{1-14} (10 or 40 µg/100 µl) or LTB (40 µg/100 µl) without Freund's adjuvant, samples were diluted in PBS and sterilized by expulsion through a 2.2 µm Millex GV sterile filter (Millipore, Bedford, MA) attached to a 5 ml syringe into a 15 ml sterile polypropylene tube.

Mice injected every 2 weeks received a primary injection of 10 or 40 µg LTB:bINHα_c^{1-14} or 40 µg LTB either in Freund's complete adjuvant or PBS followed by four subsequent boosters in Freund's incomplete or PBS. Mice injected every 4 weeks received a total of two boosters in 8 weeks. Control groups immunized against pentameric or monomeric LTB (40 µg/mouse) were injected using the 2-week injection paradigm only.

At 68 days of age, 6 weeks after the first injection (two boosters for 20 week and one booster for 4-week interval), mice were anesthetized with Metofane, bled from the orbital sinus, and plasma collected as described above. Two weeks after the final booster, at 96 days of age, all mice were anesthetized with Metofane, body weight taken, and blood collected by heart puncture as described for Example 3. Blood samples (500 to 1,500 µl/mouse) were incubated at room temperature for 2 hours and then at 4°C overnight, and serum was separated from clotted blood cells by centrifugation at 1000 x g (Beckman GPR centrifuge) for 30 minutes at 4°C and stored at -20°C until assayed. After bleeding, testis, epididymis, seminal vesicle, heart, kidney, liver and spleen for each mouse were weighed.

Antibody Titer Determination

I. Pentameric EtxB Antibody Titer

Probind microtiter plates (Falcon, Lincoln Park, NJ) were coated overnight at room temperature with 0.2 µg/well GM1 in PBS followed by incubation with 100 ng/well pentameric LTB. After blocking non-specific binding sites with

1% bovine serum albumin (BSA) dissolved in PBS-T, the ELISA plates were incubated with mouse serum diluted 1:5000 in PBS-T containing 0.1% BSA (PBS-T-B, 1 hour), horseradish peroxidase-labeled goat anti-rabbit IgG (GAM0HRP, 1 hour; Vector, Burlingame, CA) diluted 1:5000 in PBS-T-B, then ortho-phenylenediamine in 0.1M citrate buffer, pH 4.5, containing 0.01% H_xO_2 (OPD-H_2O_2) as described in Example 4. Titer = A_{490}.

Monomeric EtxB Antibody Titer

Xenobind microtiter plates (Xenopore Inc., Hawthorne, NJ) were incubated with 200 ng/well monomeric LTB in PBS overnight (room temperature) to covalently link antigen to ELISA plate wells, as recommended by the manufacturer. After blocking non-specific binding sites with 3% gelatin in PBS-T, ELISA plates were incubated with mouse serum diluted 1:5000 in PBS-T-B (1 hour), GAM-HRP (1 hour), then OPD-H_2O_2, as described for Example 4. Titer = A_{490}.

Inhibin Antibody Titer

Xenobind microtiter plates were incubated with 1 μg/well $bINH\alpha_c^{1-26}$ gly.tyr. or 1 μg/well partially purified bovine inhibin (ppbINH) in PBS overnight (room temperature) to covalently link antigen to ELISA plate wells, as recommended by the manufacturer. After blocking non-specific binding sites with 3% gelatin in PBS-T, ELISA plates were incubated with mouse plasma or serum diluted 1:100 in PBS-T-B (2 hours), GAM-HRP (1 hour), then OPD-H_2O_2, as described for Example 4. Titer = A_{490}.

FSH and LH RIA

Concentrations of FSH or LH in serum or plasma were determined in duplicate samples in RIA, as described for Example 4. Samples were analyzed in a single assay for each hormone. rFSH and rLH assay sensitivities were 0.625 and 0.156 ng/ml and intra-assay coefficients of variation (cv) were 6.3 and 1.6%, respectively. The cross reaction of FSH with LH and LH with FSH is <2% (per NIDDK guidelines).

Testosterone RIA

Concentrations of testosterone in serum were determined using the Coat-A-Count Total Testosterone assay kit from Diagnostic Products (DPC, Los Angeles, CA), per manufacturer's instruction for a non-extraction assay. Briefly, 50 µl of mouse serum was incubated with 1 ml tracer in antiserum-coated tubes at 37°C for 3 hours. The coated tubes were decanted, and radioactivity bound to the dried tubes determined in a MACC Micromedic γ-counter. All samples were analyzed in a single assay. Assay sensitivity was 0.2 ng/ml and intraassay CV was 1.2%. The cross reactivities are: estradiol = 0.02%; 5α-dihydrotestosterone = 3.4%; and other steroids = <1% (per DPC guidelines).

Statistics

Results were subjected to ANOVA. Whether significant ($P<0.05$) differences existed between means was determined by Student's *t*-test.

Results

As shown in Figures 7 and 8, active immunization of animals with either the monomeric or pentameric form of the fusion protein LTB:bINHα_c^{1-14} with Freund's adjuvant resulted in increased anti-inhibin antibody titers as compared with the LTB control, and also resulted in increased levels of FSH and LH. Testosterone levels were decreased (data not shown). In contrast, active immunization with the fusion protein in the absence of adjuvant failed to alter anti-inhibin antibody titers or reproductive hormone levels.

The invention has been described with reference to various specific and preferred embodiments and techniques. However, it should be understood that many variations and modifications may be made while remaining within the spirit and scope of the invention. All publications and patent applications in this specification are indicative of the level of ordinary skill in the art to which this invention pertains.

SEQUENCE LISTING

(1) GENERAL INFORMATION

(i) APPLICANT: Michigan State University

(ii) TITLE OF THE INVENTION: CHIMERIC LTB VACCINES

(iii) NUMBER OF SEQUENCES: 12

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Merchant, Gould, Smith, Edell, Welter & Schmidt
(B) STREET: 3100 Norwest Center, 90 South Seventh St
(C) CITY: Minneapolis
(D) STATE: MN
(E) COUNTRY: USA
(F) ZIP: 55402

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Diskette
(B) COMPUTER: IBM Compatible
(C) OPERATING SYSTEM: DOS
(D) SOFTWARE: FastSEQ Version 1.5

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: Unassigned
(B) FILING DATE: 12-NOV-1997
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: 08/747,410
(B) FILING DATE: 12-NOV-1996

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Kettelberger, Denise M
(B) REGISTRATION NUMBER: 33,924
(C) REFERENCE/DOCKET NUMBER: 11526.1-WO-01

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 612/371-5268
(B) TELEFAX: 612/332-9081
(C) TELEX:

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 587 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:
(ix) FEATURE:

(A) NAME/KEY: Coding Sequence
(B) LOCATION: 16...387
(D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
AATTCGGGAT GAATT ATG AAT AAA GTA AAA TTT TAT GTT TTA TTT ACG GCG      51
                 Met Asn Lys Val Lys Phe Tyr Val Leu Phe Thr Ala
                  1               5                  10

TTA CTA TCC TCT CTA TGT GCA CAC GGA GCT CCT CAG TCT ATT ACA GAA       99
Leu Leu Ser Ser Leu Cys Ala His Gly Ala Pro Gln Ser Ile Thr Glu
        15                  20                  25

CTA TGT TCG GAA TAT CAC AAC ACA CAA ATA TAT ACG ATA AAT GAC AAG      147
Leu Cys Ser Glu Tyr His Asn Thr Gln Ile Tyr Thr Ile Asn Asp Lys
    30                  35                  40

ATA CTA TCA TAT ACG GAA TCG ATG GCA GGC AAA AGA GAA ATG GTT ATC      195
Ile Leu Ser Tyr Thr Glu Ser Met Ala Gly Lys Arg Glu Met Val Ile
45                  50                  55                  60

ATT ACA TTT AAG AGC GGC GCA ACA TTT CAG GTC GAA GTC CCG GGC AGT      243
Ile Thr Phe Lys Ser Gly Ala Thr Phe Gln Val Glu Val Pro Gly Ser
                65                  70                  75

CAA CAT ATA GAC TCC CAA AAA AAA GCC ATT GAA AGG ATG AAG GAC ACA      291
Gln His Ile Asp Ser Gln Lys Lys Ala Ile Glu Arg Met Lys Asp Thr
            80                  85                  90

TTA AGA ATC ACA TAT CTG ACC GAG ACC AAA ATT GAT AAA TTA TGT GTA      339
Leu Arg Ile Thr Tyr Leu Thr Glu Thr Lys Ile Asp Lys Leu Cys Val
        95                  100                 105

TGG AAT AAT AAA ACC CCC AAT TCA ATT GCG GCA ATC AGT ATG GAA AAC TA  389
Trp Asn Asn Lys Thr Pro Asn Ser Ile Ala Ala Ile Ser Met Glu Asn
    110                 115                 120

GTTTGCTTTA AAAGCATGTC TAATGCTAGG AACCTATATA ACAACTACTG TACTTATACT   449
AATGAGCCTT ATGCTGCATT TGAAAAGGCG GTAGAGGATG CAATACCGAT CCTTAAACTG   509
TAACACTATA ACAGCTTCCA CTACAGGGAG CTGTTATAGC AAACAGAAAA AACTAAGCTA   569
GGCTGGGGGG GCAAGCTT                                                587
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 124 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE: internal
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Met Asn Lys Val Lys Phe Tyr Val Leu Phe Thr Ala Leu Leu Ser Ser
 1               5                  10                  15
Leu Cys Ala His Gly Ala Pro Gln Ser Ile Thr Glu Leu Cys Ser Glu
            20                  25                  30
Tyr His Asn Thr Gln Ile Tyr Thr Ile Asn Asp Lys Ile Leu Ser Tyr
        35                  40                  45
Thr Glu Ser Met Ala Gly Lys Arg Glu Met Val Ile Ile Thr Phe Lys
    50                  55                  60
Ser Gly Ala Thr Phe Gln Val Glu Val Pro Gly Ser Gln His Ile Asp
65                  70                  75                  80
Ser Gln Lys Lys Ala Ile Glu Arg Met Lys Asp Thr Leu Arg Ile Thr
                85                  90                  95
Tyr Leu Thr Glu Thr Lys Ile Asp Lys Leu Cys Val Trp Asn Asn Lys
            100                 105                 110
Thr Pro Asn Ser Ile Ala Ala Ile Ser Met Glu Asn
        115                 120
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:
(ix) FEATURE:

(A) NAME/KEY: Coding Sequence
(B) LOCATION: 1...39
(D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
GCT CCT CAG TCT ATT ACA GAA CTA TGT TCG GAA TAT CAC                 39
Ala Pro Gln Ser Ile Thr Glu Leu Cys Ser Glu Tyr His

 1               5                  10
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE: internal
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
Ala Pro Gln Ser Ile Thr Glu Leu Cys Ser Glu Tyr His
 1               5                  10
```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:
(ix) FEATURE:

(A) NAME/KEY: Coding Sequence
(B) LOCATION: 1...39
(D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
GCT CCT CAG TCT ATT ACA GAT CTA TGT TCG GAA TAT CAC                39
Ala Pro Gln Ser Ile Thr Asp Leu Cys Ser Glu Tyr His

 1               5                  10
```

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE: internal
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
Ala Pro Gln Ser Ile Thr Asp Leu Cys Ser Glu Tyr His
 1               5                  10
```

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:
(ix) FEATURE:

(A) NAME/KEY: Coding Sequence
(B) LOCATION: 1...31
(D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```
ATG GCA GGC AAA AGA GAA ATG GTT ATC ATT A                    31
Met Ala Gly Lys Arg Glu Met Val Ile Ile Il

 1               5                  10
```

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE: internal
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

```
Xaa Xaa Gly Lys Arg Glu Met Val Ile Ile Ile
 1               5                  10
```

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:
(ix) FEATURE:

(A) NAME/KEY: Coding Sequence
(B) LOCATION: 1...31
(D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

```
ATG GCA GGC CTA AGA GAA ATG GTT ATC ATT A                              31
Met Ala Gly Leu Arg Glu Met Val Ile Ile Il
 1               5                  10
```

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE: internal
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

```
Met Ala Gly Leu Arg Glu Met Val Ile Ile Ile
 1               5                  10
```

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 66 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:
(ix) FEATURE:

(A) NAME/KEY: Coding Sequence
(B) LOCATION: 1...31
(D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

```
GTC GAA GTC CCT GGA TCC ACC CCG CCG CTG C CGTGGCCGTG GTCCCCGGCT GC    53
Val Glu Val Pro Gly Ser Thr Pro Pro Leu Pro
 1               5                  10

TCTGGGCAGT CAA                                                       66
```

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE: internal
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Val Glu Val Pro Gly Ser Thr Pro Pro Leu Pro
1 5 10

WE CLAIM:

1. A nucleic acid construct comprising:

a nucleic acid sequence encoding an antigenic peptide; and

a nucleic acid sequence encoding LTB;

wherein the sequence encoding the antigenic peptide is inserted into the sequence encoding an exposed loop of LTB, and wherein said insertion does not disrupt the reading frame of the LTB sequence.

2. The nucleic acid construct of claim 1, wherein said antigenic peptide sequence is inserted into a unique restriction site in the nucleic acid sequence encoding LTB.

3. The construct of claim 2, wherein the restriction site is *Sma*I.

4. The construct of claim 2, wherein the unique restriction site is engineered into the sequence encoding LTB.

5. The nucleic acid construct of claim 1, wherein the encoded antigenic peptide is bINHα_c^{1-14} or bINHα_c^{1-27}.

6. A fusion protein comprising:

LTB; and

an inserted peptide antigen,

wherein the inserted peptide antigen is expressed on an exposed surface loop of LTB, and wherein the fusion protein is immunoreactive with anti-inserted peptide antigen antibodies.

7. The fusion protein of claim 6, wherein the antigenic peptide is bINHα_c^{1-14} or bINHα_c^{1-26}.

8. The fusion protein of claim 6, wherein said antigenic peptide is inserted at or near *etxB* nucleotide 237.

9. The fusion protein of claim 6, wherein said LTB comprises a monomer or pentamer.

10. A composition comprising the immunogenic fusion protein of claim 6 and an adjuvant.

11. A vaccine comprising the immunogen of claim 10.

12. The vaccine of claim 11, wherein said antigenic peptide comprises $bINH\alpha_c^{1-14}$ or $bINH\alpha_c^{1-26}$.

13. A method for producing an immunogenic fusion protein comprising the steps of:

inserting a nucleic acid sequence encoding an antigenic peptide into a nucleic acid sequence encoding an exposed loop of LTB to form a fusion construct;

expressing the fusion protein in an appropriate host to generate a fusion protein presenting the antigenic peptide on an loop on the surface of LTB, wherein the fusion protein is immunoreactive with anti-inserted antigen antibodies.

14. The method of claim 13, wherein said antigenic peptide is inserted at a unique restriction site in the sequence encoding LTB.

15. The method of claim 13, wherein the restriction site is *Sma*I.

16. The method of claim 13, wherein said unique restriction site is engineered into the sequence encoding an exposed loop on LTB.

17. A multiple-antigen vaccine comprising pentameric LTB having multiple exposed protein surfaces containing a foreign antigen.

18. Pentameric LTB modified to contain antigenic peptide by insertion in the sequence encoding the pentameric LTB of a foreign nucleic acid sequence at approximately nucleotide 237.

19. A method of inducing an immune response in a host animal against a desired protein comprising the steps of:

administering to the animal a fusion protein comprising an antigenic fragment of the desired protein inserted in an exposed loop of LTB.

20. The method of claim 19, wherein said administering is by oral ingestion of the fusion protein.

21. The method of claim 19, wherein said desired protein is inhibin, said antigenic fragment is bINHα_c^{1-14} or bINHα_c^{1-27}, and said fusion protein is encoded by the nucleic acid sequence of *etxB* having a foreign nucleic acid sequence inserted at approximately nucleotide 237.

22. A method of enhancing fertility of an animal comprising the steps of:

administering to the animal a fusion protein comprising an antigenic fragment of inhibin inserted in an exposed loop of LTB.

23. The method of claim 22, wherein said antigenic peptide comprises bINHα_c^{1-14} or bINHα_c^{1-26}.

24. The method of claim 22, wherein administering the fusion protein increases levels in the animal of follicle stimulating hormone.

25. The method of claim 22, wherein administering the fusion protein enhances production of sperm or ova.

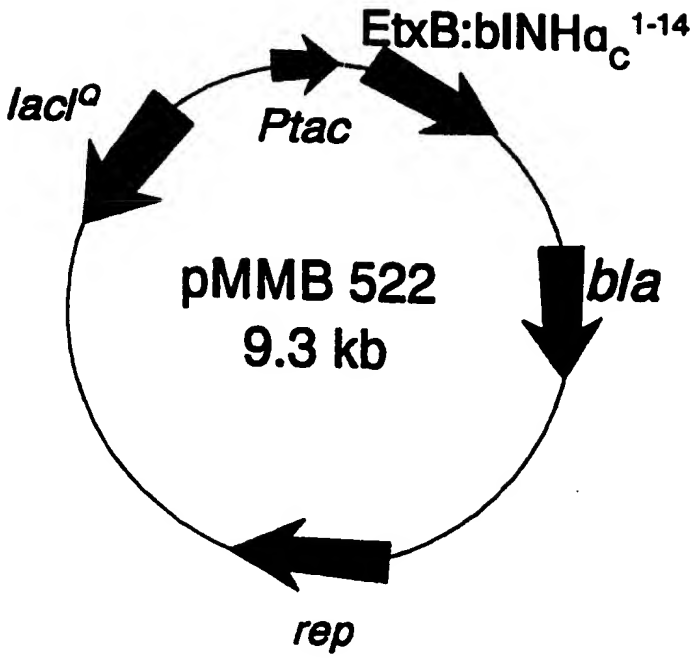


FIG. 1

FIG. 2A

Probed with Anti-EtxB

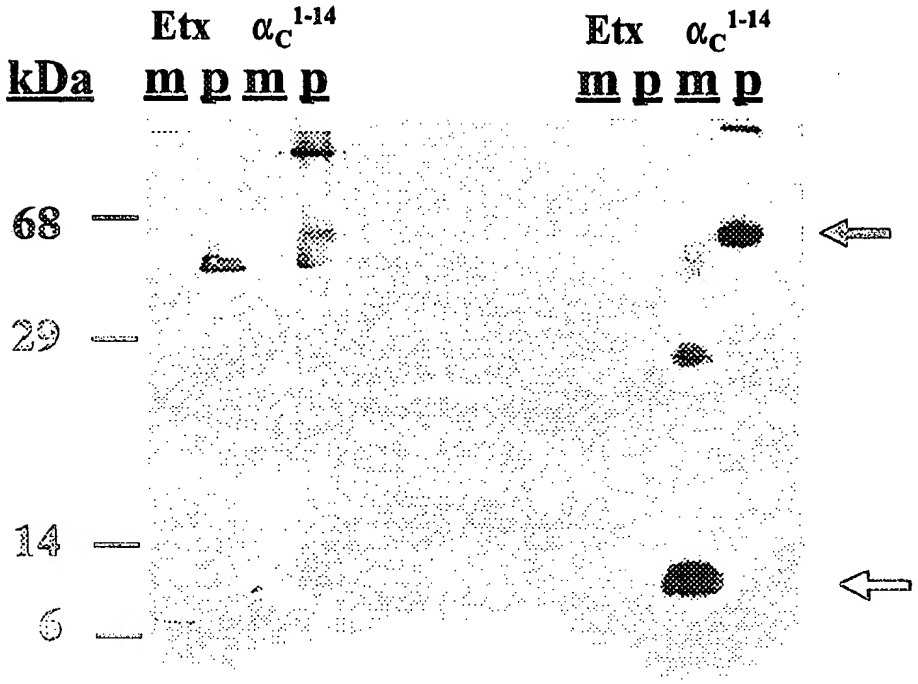


FIG. 2B

Probed with Anti-α_C^{1-26}bINH

FIG. 3

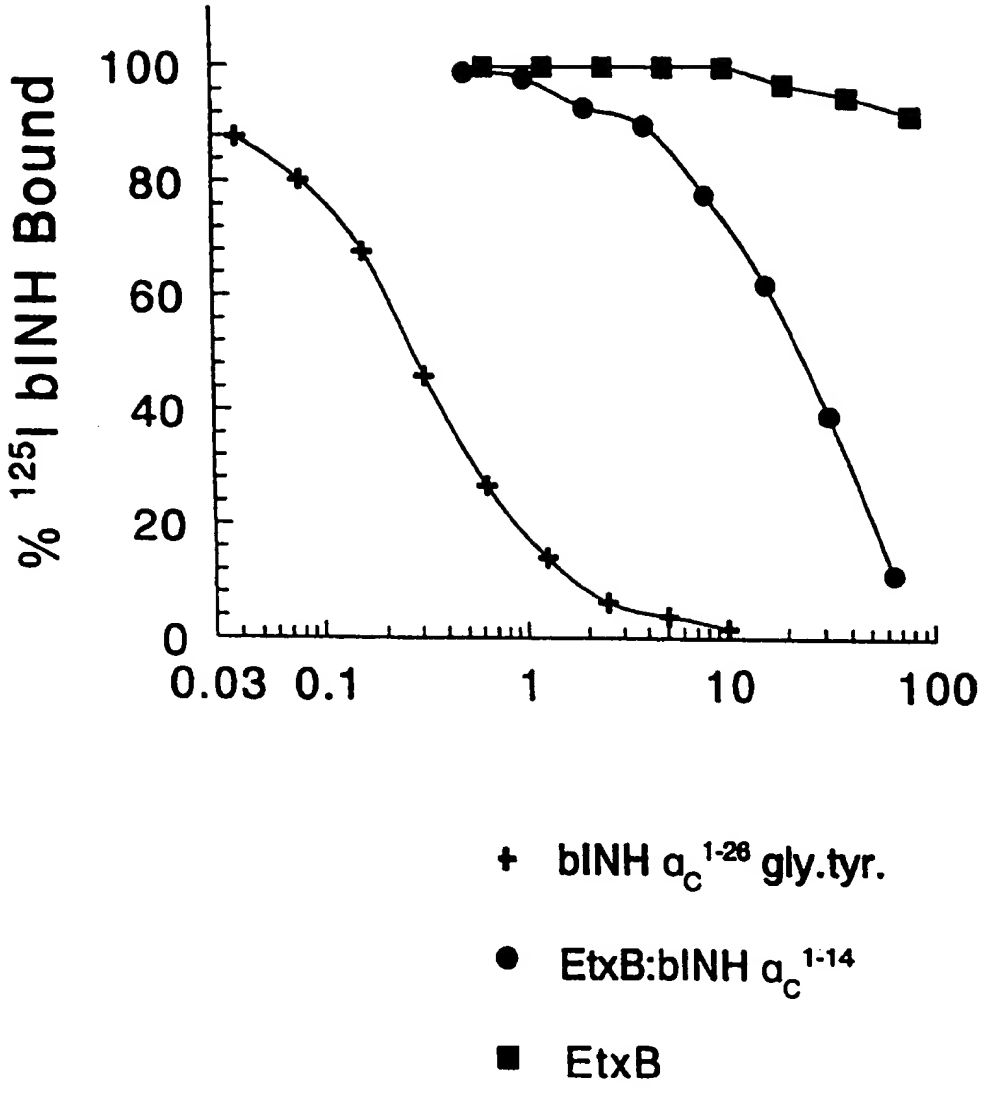
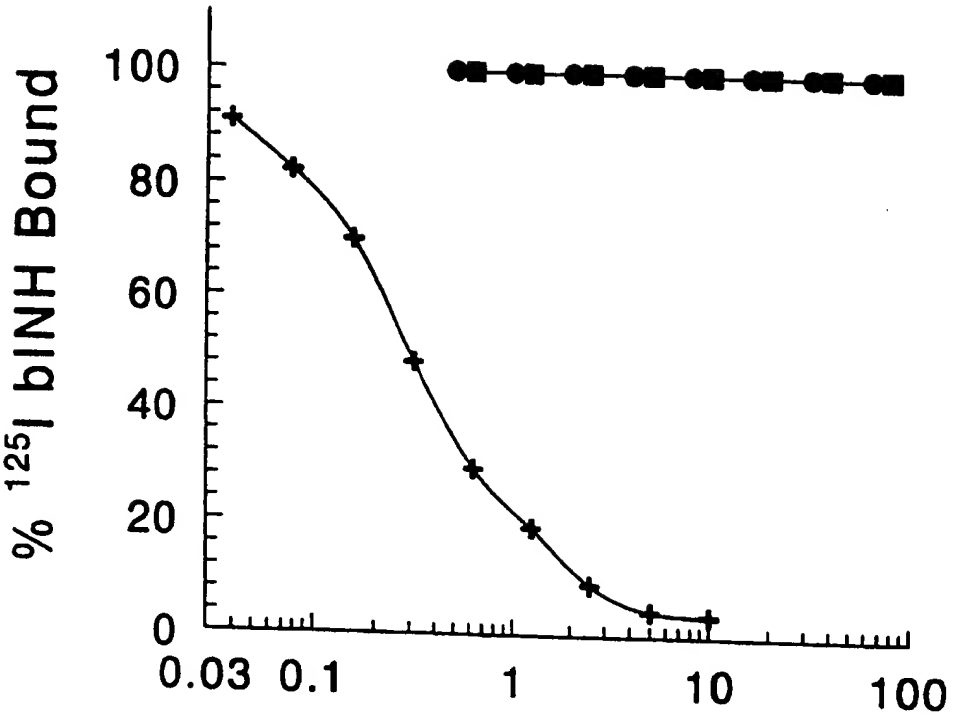

% 125I bINH Bound
100
80
60
40
20
0
0.03
0.1
1
10
100
+ bINH α_c^{1-28} gly.tyr.
● EtxB:bINH α_c^{1-14}
■ EtxB

FIG. 4



+ bINH α_c^{1-26} gly.tyr.

● EtxB:bINH α_c^{1-14}

■ EtxB

FIG. 5

Active Immunization

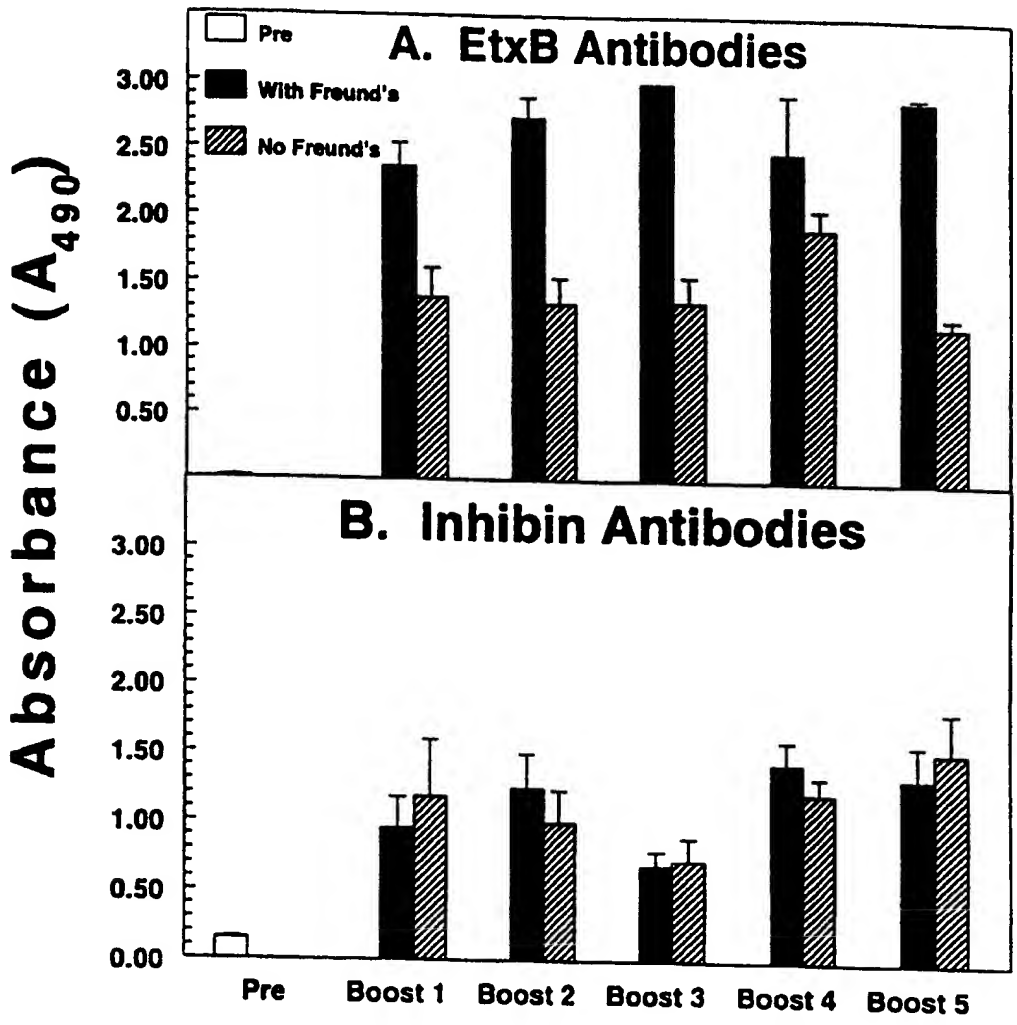


FIG. 6A

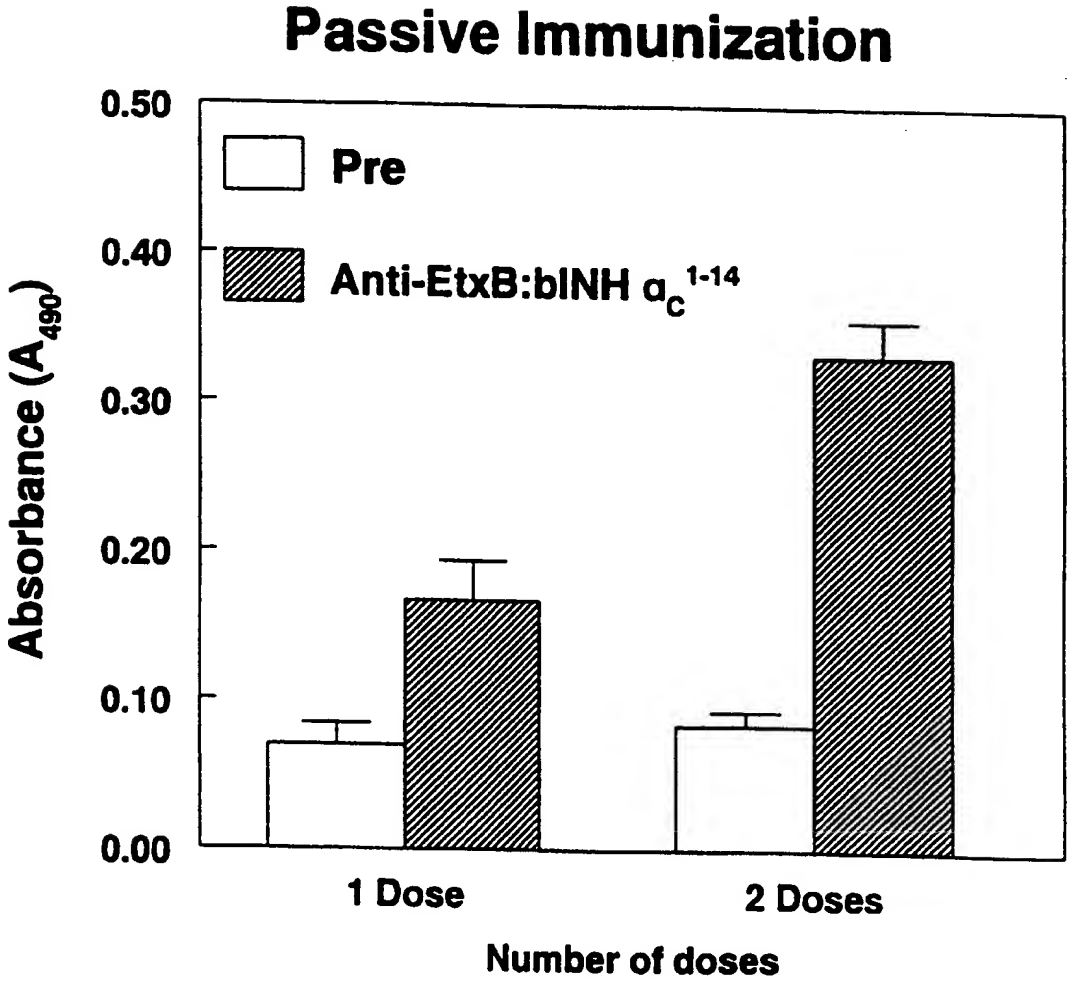

Passive Immunization
0.50
0.40
0.30
0.20
0.10
0.00
Absorbance (A_{490})
Pre
Anti-EtxB:bINH α_c^{1-14}
1 Dose
2 Doses
Number of doses

FIG. 6B

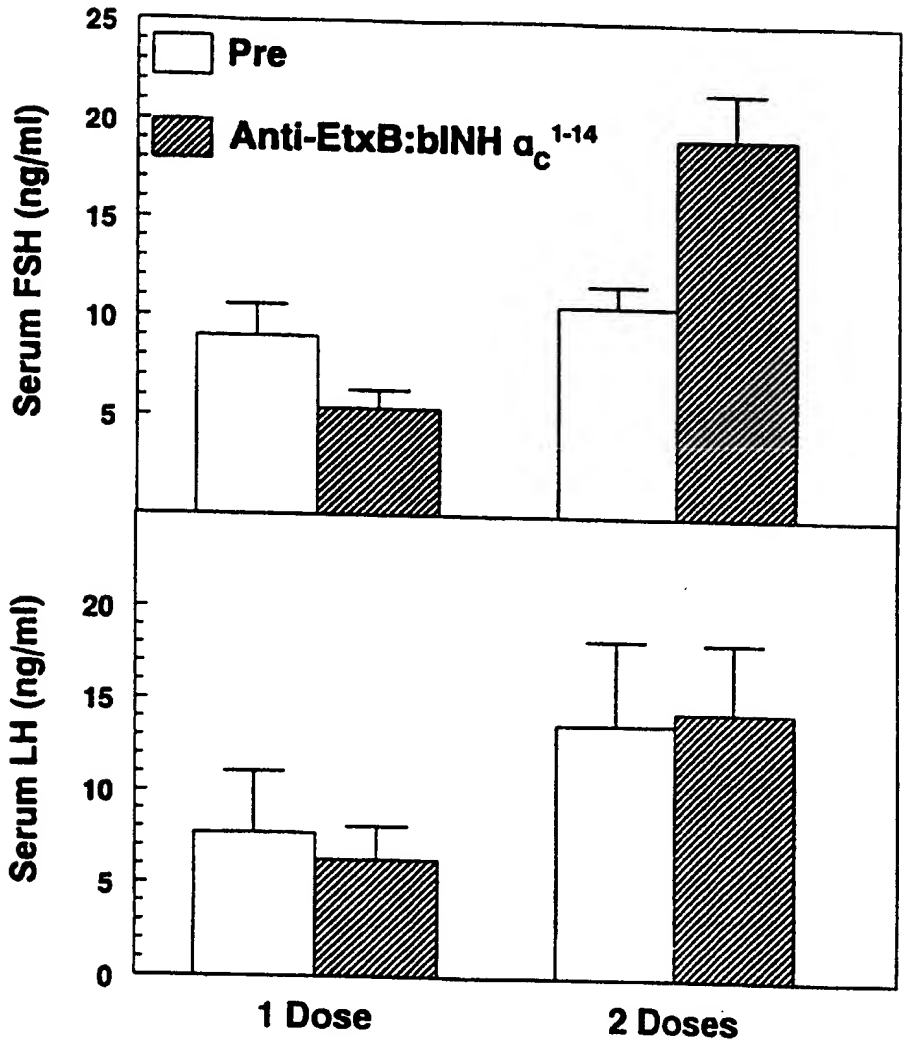

Passive Immunization
Pre
Anti-EtxB:bINH α_c^{1-14}
Serum FSH (ng/ml)
25
20
15
10
5
Serum LH (ng/ml)
20
15
10
5
0
1 Dose
2 Doses
Number of Doses

FIG. 6C

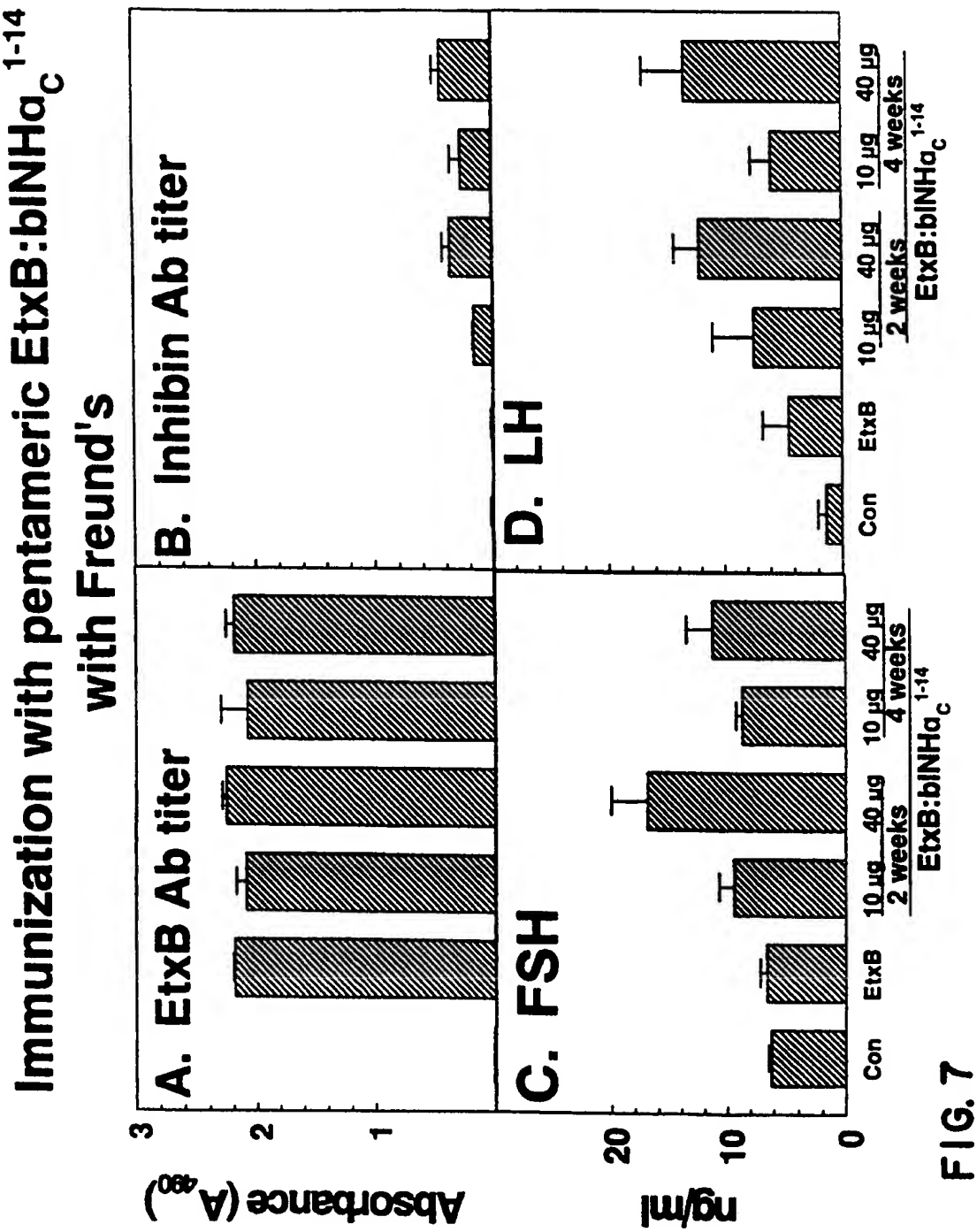

Immunization with pentameric EtxB:blNHα$_C^{1-14}$ with Freund's
A. EtxB Ab titer
B. Inhibin Ab titer
C. FSH
D. LH
Absorbance (A$_{490}$)
ng/ml
3
2
1
20
10
0
Con
EtxB
10 µg 2 weeks EtxB:blNHα$_C^{1-14}$
40 µg 2 weeks EtxB:blNHα$_C^{1-14}$
10 µg 4 weeks EtxB:blNHα$_C^{1-14}$
40 µg 4 weeks EtxB:blNHα$_C^{1-14}$

FIG. 7

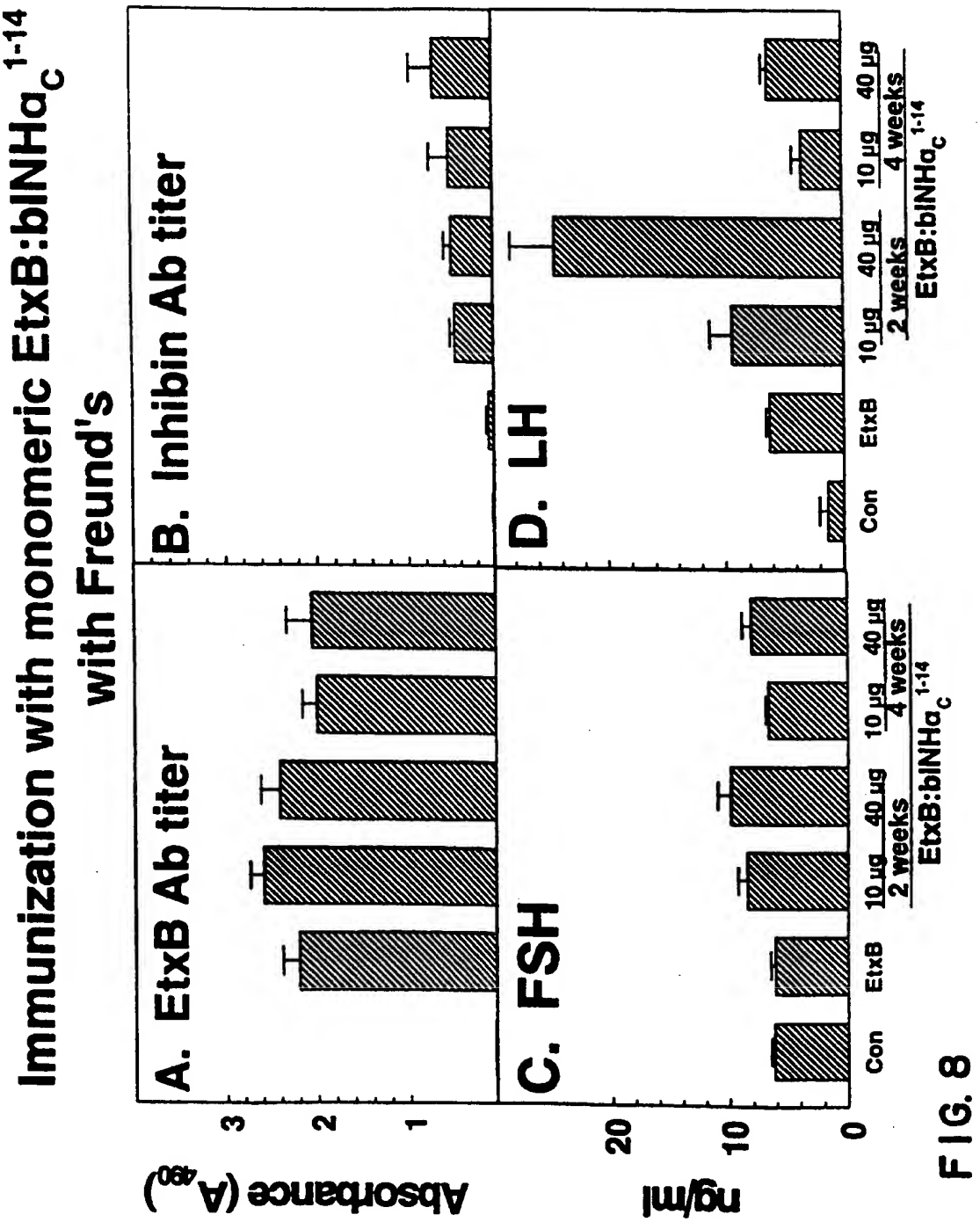

Immunization with monomeric EtxB:blNHα$_C^{1-14}$ with Freund's
A. EtxB Ab titer
B. Inhibin Ab titer
C. FSH
D. LH
Absorbance (A$_{490}$)
ng/ml
0
10
20
1
2
3
Con
EtxB
10 µg 40 µg 2 weeks
10 µg 40 µg 4 weeks
EtxB:blNHα$_C^{1-14}$

FIG. 8

FIG. 9

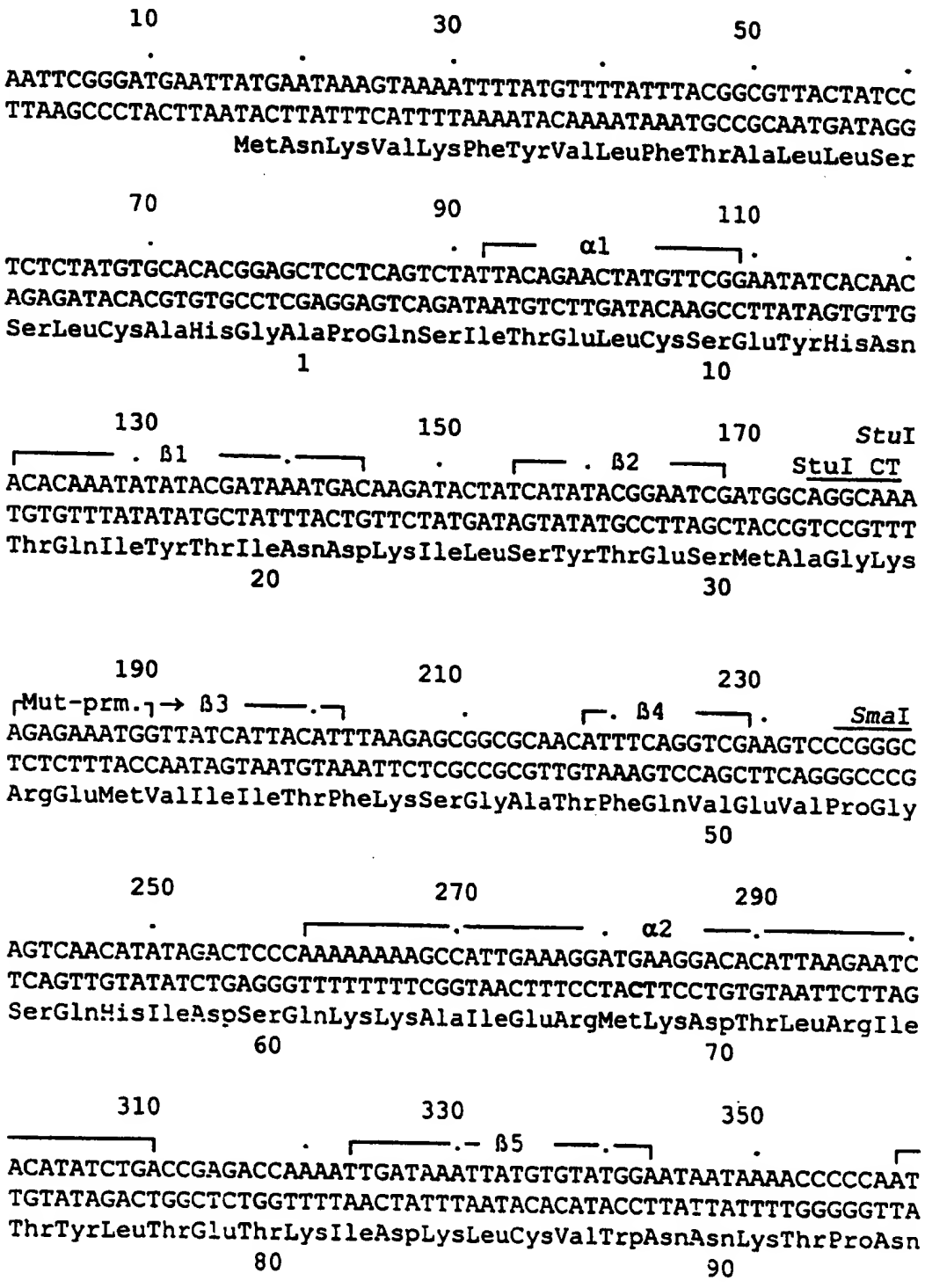

10
30
50
AATTCGGGATGAATTATGAATAAAGTAAAATTTTATGTTTTATTTACGGCGTTACTATCC
TTAAGCCCTACTTAATACTTATTTCATTTTAAAATACAAAATAAATGCCGCAATGATAGG
MetAsnLysValLysPheTyrValLeuPheThrAlaLeuLeuSer
70
90
110
α1
TCTCTATGTGCACACGGAGCTCCTCAGTCTATTACAGAACTATGTTCGGAATATCACAAC
AGAGATACACGTGTGCCTCGAGGAGTCAGATAATGTCTTGATACAAGCCTTATAGTGTTG
SerLeuCysAlaHisGlyAlaProGlnSerIleThrGluLeuCysSerGluTyrHisAsn
1
10
130
150
170
StuI
ß1
ß2
StuI CT
ACACAAATATATACGATAAATGACAAGATACTATCATATACGGAATCGATGGCAGGCAAA
TGTGTTTATATATGCTATTTACTGTTCTATGATAGTATATGCCTTAGCTACCGTCCGTTT
ThrGlnIleTyrThrIleAsnAspLysIleLeuSerTyrThrGluSerMetAlaGlyLys
20
30
190
210
230
Mut-prm.
ß3
ß4
SmaI
AGAGAAATGGTTATCATTACATTTAAGAGCGGCGCAACATTTCAGGTCGAAGTCCCGGGC
TCTCTTTACCAATAGTAATGTAAATTCTCGCCGCGTTGTAAAGTCCAGCTTCAGGGCCCG
ArgGluMetValIleIleThrPheLysSerGlyAlaThrPheGlnValGluValProGly
50
250
270
290
α2
AGTCAACATATAGACTCCCAAAAAAAAGCCATTGAAAGGATGAAGGACACATTAAGAATC
TCAGTTGTATATCTGAGGGTTTTTTTTCGGTAACTTTCCTACTTCCTGTGTAATTCTTAG
SerGlnHisIleAspSerGlnLysLysAlaIleGluArgMetLysAspThrLeuArgIle
60
70
310
330
350
ß5
ACATATCTGACCGAGACCAAAATTGATAAATTATGTGTATGGAATAATAAAACCCCCAAT
TGTATAGACTGGCTCTGGTTTTAACTATTTAATACACATACCTTATTATTTTGGGGGTTA
ThrTyrLeuThrGluThrLysIleAspLysLeuCysValTrpAsnAsnLysThrProAsn
80
90

FIG. 9 (Cont'd)

```
        370                 390                 410
—— ß6  .  ———— .  —┐ SpeI      .         .         .
TCAATTGCGGCAATCAGTATGGAAAACTAGTTTGCTTTAAAAGCATGTCTAATGCTAGGA
AGTTAACGCCGTTAGTCATACCTTTTGATCAAACGAAATTTTCGTACAGATTACGATCCT
SerIleAlaAlaIleSerMetGluAsnEnd
                100

        430                 450                 470
         .         .         .         .         .         .
ACCTATATAACAACTACTGTACTTATACTAATGAGCCTTATGCTGCATTTGAAAAGGCGG
TGGATATATTGTTGATGACATGAATATGATTACTCGGAATACGACGTAAACTTTTCCGCC

        490                 510                 530
TAGAGGATGCAATACCGATCCTTAAACTGTAACACTATAACAGCTTCCACTACAGGGAGC
ATCTCCTACGTTATGGCTAGGAATTTGACATTGTGATATTGTCGAAGGTGATGTCCCTCG

         550                 570
  .         .         .         .         .HindIII
TGTTATAGCAAACAGAAAAAACTAAGCTAGGCTGGGGGGGCAAGCTT
ACAATATCGTTTGTCTTTTTTGATTCGATCCGACCCCCCCGTTCGAA
```

INTERNATIONAL SEARCH REPORT

Intern ial Application No
PCT/US 97/20584

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/62 C12N15/31 C07K14/245 C07K14/575 A61K38/22
A61K39/108

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 93 22450 A (UNIV CALIFORNIA ;SERAGEN INC (US)) 11 November 1993 see page 9 see page 12 - page 14 see page 21 - page 22; claims 1-27 ---	1,2,4,6, 9-11,13, 14,17
Y	DALLAS W ET AL: "Amino acid sequence homology between cholera toxin and Escherichia coli heat-labile toxin" NATURE., vol. 288, 1980, LONDON GB, pages 499-501, XP002058571 see abstract; figure 3 --- -/--	1,2,4,6, 9-11,13, 14,17

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
11 March 1998	27.03.98

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Espen, J

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Interna al Application No
PCT/US 97/20584

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SIXMA T K ET AL: "Crystal structure of a cholera toxin-related heat-labile enterotoxin from E. coli" NATURE., vol. 351, 1991, LONDON GB, pages 371-377, XP002058572 cited in the application see page 374 see page 377, right-hand column	1,2,4,6, 9-11,13, 14,17
Y	VERLINDE CL ET AL: "Protein crystallography and infectious diseases." PROTEIN SCI, OCT 1994, 3 (10) P1670-86, UNITED STATES, XP002058573 see page 1678 - page 1684	1,2,4,6, 9-11,13, 14,17
Y	MICHEL LO ET AL: "Specificity of the protein secretory apparatus: secretion of the heat-labile enterotoxin B subunit pentamers by different species of gram-bacteria." GENE, JAN 11 1995, 152 (1) P41-5, NETHERLANDS, XP004042585 see figure 1	1,2,4,6, 9-11,13, 14,17
Y	MARCELLO A ET AL: "Efficient extracellular production of hybrid E. coli heat-labile enterotoxin B subunits in a marine Vibrio." FEMS MICROBIOL LETT, MAR 15 1994, 117 (1) P47-51, NETHERLANDS, XP002058574 see abstract	1,2,4,6, 9-11,13, 14,17
Y	MASON H S ET AL: "TRANSGENIC PLANTS AS VACCINE PRODUCTION SYSTEMS" TRENDS IN BIOTECHNOLOGY, vol. 13, no. 9, September 1995, pages 388-392, XP002024035 cited in the application see page 389, right-hand column - page 391, left-hand column	1,2,4,6, 9-11,13, 14,17
Y	NASHAR TO ET AL: "Current progress in the development of the B subunits of cholera toxin and Escherichia coli heat-labile enterotoxin as carriers for the oral delivery of heterologous antigens and epitopes." VACCINE, 1993, 11 (2) P235-40, ENGLAND, XP000645274 see page 236	1,2,4,6, 9-11,13, 14,17
A	WO 88 00208 A (SALK INST FOR BIOLOGICAL STUDI) 14 January 1988	

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 97/20584

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

see FURTHER INFORMATION sheet PCT/ISA/210

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 97/20584

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Remark : Although claims 19-26 are directed to a method of treatment of the human/animal body , the search has been carried out and based on the alleged effects of the compound/composition.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Interna al Application No
PCT/US 97/20584

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9322450 A	11-11-93	AU 4238893 A	29-11-93
WO 8800208 A	14-01-88	AU 605162 B	10-01-91
		AU 7640887 A	29-01-88
		EP 0272309 A	29-06-88
		JP 1500121 T	19-01-89
		US 5015729 A	14-05-91